The Development of Antimicrobial Resistance in *Acinetobacter* spp and Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Background: *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA) represent the most worrying Gram-negative and Gram-positive nosocomial pathogens of the present age. They are of increasing concern in the clinical environment due to their multi-drug resistance and the dwindling therapeutic options available. *A. baumannii* is the most frequently isolated clinical species of the genus, and is able to rapidly acquire resistance. Hypermutators, most frequently deficient in mismatch repair (MMR) via defects in the *mutS* gene, have been associated with antimicrobial resistance in several bacterial populations. To date, however, the potential role of MMR-deficient mutators in the development of resistance in clinical *Acinetobacter* spp. has not been investigated. Biocides, most notably chlorhexidine (CHX), are increasingly used in the hospital environment to prevent bacterial spread. This has led to concerns about the development of reduced biocide susceptibility and associated antibiotic resistance in hospital bacterial populations, where there is frequent exposure to both of these factors. The effect of CHX upon defined clinical MRSA isolates is examined here.

Methods: The *mutS* gene of clinical *Acinetobacter* spp. isolates with varying sensitivities was sequenced and compared to establish whether any variations were present. Mutation studies were performed on isolates by challenging them with ciprofloxacin to determine whether different *mutS* types correlated with any variation in their ability to develop significant fluoroquinolone resistance. The response of clinical MRSA isolates to a range of CHX concentrations was examined with susceptibility

testing methods, and effects were compared with standard strains. Determination of post-exposure minimum inhibitory concentrations (MICs) of a range of antibiotics enabled evaluation of whether exposure to CHX had an effect on susceptibility to antibiotics.

Results: Variation was observed in the *mutS* gene of clinical *Acinetobacter* spp. isolates, with greater homology observed as resistance increased. A highly conserved and previously unreported amino acid sequence was discovered in resistant isolates. Non-resistant isolates with this 'R-type' *mutS* sequence appeared to have a greater ability to develop significant ciprofloxacin resistance. Clinical MRSA isolates had varying susceptibility to CHX, and there were differences in the susceptibility of standard strains compared to clinical isolates. CHX residues exerted a prolonged minimal inhibitory effect, and several increases in antibiotic MICs following CHX exposure were observed.

Conclusions: The correlation of the *mutS* sequence with mutation ability suggests that defects in the *mutS* gene may have a role to play in the ability of certain *Acinetobacter* spp. to rapidly acquire resistance. This could have implications for the treatment of *Acinetobacter* spp. infections, and may enable quick determination of which clinical isolates have the potential to develop clinically significant resistance. Incomplete eradication due to the prolonged minimal effect of CHX residues may act as a selective pressure in the hospital environment, allowing survival of reduced susceptibility MRSA isolates. Increases in antibiotic MICs following CHX exposure is of grave concern for the future of biocide usage.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated, and it has not been submitted for any other degree or professional qualification:

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Publications and Presentations

1. Davies, S. E., Brown, S. & Amyes, S. G. B. (2004). Correlation of the mutation potential of *Acinetobacter baumannii* clinical isolates with alterations in the *mutS* gene (14th European Congress of Clinical Microbiology and Infectious Diseases. P1735). *Clinical Microbiology and Infection.* 10(suppl 3), 492.

2. Davies, S. E., Brown, S. & Amyes, S. G. B. (2004). Correlation of the mutation potential of *Acinetobacter baumannii* clinical isolates with alterations in the *mutS* gene. 6th International Symposium on the Biology of Acinetobacter. Poster presentation.

3. Davies, S. E., Vali, L., & Amyes, S. G. B. (2006). The effect of biocide residues on survival of methicillin resistant *Staphylococcus aureus*. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, K-1186.

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Abbreviations

aa	amino acid
Acb	*Acinetobacter calcoaceticus-Acinetobacter baumannii* complex
AIDS	Acquired Immuno-Deficiency Syndrome
AFLP	Amplified Fragment Length Polymorphism
ARDRA	amplified 16S ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
bp	base pair
BSAC	British Society for Antimicrobial Chemotherapy
CA-MRSA	community-acquired/community-associated MRSA
CHX	chlorhexidine
cfu	colony forming units
CIP	ciprofloxacin
DNA	deoxyribonucleic acid
EMRSA	epidemic methicillin-resistant *Staphylococcus aureus*
gen.sp.	genomic species
gyrA	gene encoding the GyrA subunit of DNA Gyrase
HA-MRSA	hospital-acquired MRSA
HIV	Human Immuno-deficiency Virus
HPA	Health Protection Agency
kb	kilobase
MDR	Multi-drug Resistant

ME	microbiocidal effect
MIC	minimum inhibitory concentration
MMR	Mismatch Repair
MRAb	Multi-resistant *Acinetobacter baumannii*
MRSA	methicillin-resistant *Staphylococcus aureus*
mutS	gene encoding the MutS bacterial protein involved in MMR
NCTC	National Collection of Type Cultures
NRIE	New Royal Infirmary Edinburgh
nuc	nucleotide
OMP	outer membrane protein
parC	gene encoding the ParC subunit of DNA Topoisomerase IV
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
QRDR	Quinolone Resistance Determining Region
RFLP	Restriction Fragment Length Polymorphism
RIE	Royal Infirmary Edinburgh
RNA	ribonucleic acid
R-type	*mutS* aa sequence found in resistant *Acinetobacter* in this study
SARS	Severe Acute Respiratory Syndrome
SDW	sterile distilled water
spp.	species
tRNA	transfer RNA
UTI	urinary tract infection

Contents

Chapter 1 - Introduction

1.1 Nosocomial Pathogens and Problems of Resistant Bacteria

1.1.1 The Antibiotic Era

The accidental discovery of the antibiotic properties of penicillin in the late 1920s by Sir Alexander Fleming (Fleming, 1929), followed by its development and use as a chemotherapeutic agent in the 1940s (Chain *et al*, 1940) began a new age in the war between clinicians and infectious diseases: the antibiotic era.

The discovery and development of new antibiotics led to their widespread use, with the development of many different classes of antibiotics, seen as safe and effective targeted treatment against the specific causes of infection. They were initially regarded as 'wonder drugs' and a cure-all treatment, often used for even minor infections, some non-bacterial. An increasing number of infectious diseases seemed within the clinician's control and it was hoped that continued antibiotic development could lead to the eradication of infectious diseases.

However, the current situation is very far from this ideal picture; since the introduction of the first antibiotics, bacteria have in turn evolved resistance mechanisms to counteract the attacks of these drugs.

Even in the early days of antibiotic use, there was an awareness of developing resistance to these agents, particularly in common hospital bacteria of the time; there were increasing reports of penicillin-resistant *Staphylococcus pyogenes* in the mid-1940s, compared to very few before 1944 (Barber & Rozwadowska-Dowzenko, 1948). It could not have been known then that the development, use, and subsequent overuse of antibiotics would escalate to what has been likened to an arms race between bacteria and the drugs used against them brought about by the rise in antibiotic resistance in first Gram-positive and then Gram-negative bacteria.

1.1.2 The Rise of Antibiotic Resistance

With increasing resistance apparent, more classes of antibiotics and iterations of existing compounds were developed to replace those that were failing; this continued ability to treat infections effectively kept the problem of antibiotic resistance at bay, as treatment options were not affected. However, the lack of development of new classes of antibiotics and reliance, instead, upon iterations of existing classes, has meant that treatment is now compromised (Amyes, 2000). It now seems clear that antibiotics will not live up to the hopes they once inspired for the eradication of infectious diseases. Indeed, there are new and recent emerging infectious diseases such as AIDS (Acquired Immuno-Deficiency Syndrome) and SARS (Severe Acute Respiratory Syndrome) to contend with, in addition to increasing multi-drug resistance in bacteria leading to the re-emergence of previously well-controlled infections, such as *Mycobacterium tuberculosis* (Gillespie, 2002).

Of particular concern is the prevalence of multi-drug resistant (MDR) strains, not only of traditionally important bacteria in the clinical environment such as methicillin-resistant *Staphylococcus aureus* (MRSA), but also of bacteria never previously associated with infection, such as *Acinetobacter baumannii*, including isolates resistant to all routinely used available antibiotics (Kuo *et al*, 2004), leaving the clinician with few treatment options.

The adaptation of bacteria to their environmental stresses is a Darwinian 'survival of the fittest' scenario, where those of the population able to survive the pressure are those that are able to reproduce and give rise to the subsequent generation. Bacteria are advantaged in this evolution not only by their large numbers and fast replication rate in comparison to higher organisms, but also in their ability to readily acquire and exchange genetic information, thereby introducing variation (Wise, 2004), in which hypermutation may play a role (as detailed in Section 1.5).

Briefly, antibiotic resistance will develop in a population where at least one bacterium exists which is able to survive the presence of that antibiotic. The initial development of resistance involves random production of new combinations of genes and strains, but this in itself is not enough to drive resistance development; instead interplay of complex factors favours survival and proliferation of a particular combination at that time (Livermore, 2007). It is the combination of resistance genes being developed and the selective pressure exerted by antibiotics that can lead to proliferation of these survivors and the antibiotic resistance problem which is now evident. Continued selective pressure

of the antibiotic challenge can lead to resistant phenotypes from an originally heterogenous population becoming predominant over the course of several generations. As such, antibiotic resistance has only become a problem since the increased use of antibiotics. However, even in the absence of the selecting antibiotic, resistant bacteria may remain (Levy, 2002a).

The changes that may lead to resistance arising in a bacterial population vary depending on the mode of action of the antibiotic, and also upon the target bacteria. There are several major classes of antibiotic, with various modes of action. Table 1.1 provides examples of the modes of action of some of the major antibiotic classes. For each of these classes however, there now exists at least one and often several mechanisms of resistance.

Table 1.1 The modes of action of major antibiotic classes

Adapted from Levy & Marshall, 2004.

Mode of Action	Antibiotic Families
Inhibition of cell wall synthesis	β-lactams (penicillins, cephalosporins, carbapenems, monobactams) Glycopeptides Cyclic lipopeptides
Inhibition of protein synthesis	Tetracyclines Aminoglycosides Oxazolidonones (Linezolid) Streptogramins Ketolides Macrolides Lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Inhibition of RNA synthesis	Rifampicin
Competitive inhibition of folic acid synthesis	Sulfonamides Trimethoprim
Membrane disorganising agents	Polymyxins (Polymyxin-B, Colistin)

Bacterial resistance arises through the development of variation via horizontal transfer or mutation. Horizontal transfer includes the acquisition of resistance genes from other organisms carried on plasmids, transposons and integrons, mediated by conjugation, transduction or transformation; foreign DNA may also be incorporated into the chromosome by recombination. Mutations in chromosomal loci relevant to antibiotic resistance development include those leading to changes in genes encoding the antibiotic target, alterations in the expression of intrinsic resistance mechanisms and overproduction of antibiotic inactivating enzymes (some examples are detailed in Section 1.5.2) (Spratt, 1994; Mazel & Davies, 1999; de la Cruz & Davies, 2000). The importance of mutation in resistance development is discussed in Section 1.5, and fluoroquinolone resistance is detailed in Section 1.4.

Whilst the factors influencing the development of resistance, and the complexities of resistance development itself, are often uncertain, it is clear that the selective pressure exerted by antibiotic use is a major factor driving this development (Barbosa & Levy, 2000).

1.1.3 Multi-drug Resistance

Multi-drug resistant (MDR) bacteria are a serious problem, particularly in the hospital environment where the increased use and presence of antibiotics compared to the community can create a greater selective pressure. Reports of ‘pan-drug’ resistant strains of bacteria, resistant to all commonly used agents including last-resort treatment options,

has serious implications for the continued ability to treat infections caused by these bacteria and has led to the re-use of and reliance upon toxic agents such as polymyxin as therapy (Falagas and Bliziotis, 2007; Meyer, 2005).

1.1.4 Impacts of MDR Bacteria in the Hospital Environment

Patients and healthcare workers within the hospital may be colonised by bacteria which may also be subjected to these selective pressures and develop into MDR bacteria. As such, colonised patients can act as a reservoir of MDR bacteria and immuno-compromised patients, as found in the Intensive Care Unit (ICU), are particularly vulnerable to subsequent infection (Walsh & Amyes, 2004). This has led to opportunistic pathogens such as *Acinetobacter* spp becoming an increasing problem. Additionally, several bacteria have been found to persist in the hospital environment. For example, *Acinetobacter* spp have been found on many surfaces, from intravascular catheters to curtains (Das *et al*, 2002) and are able to survive for long periods (Jawad *et al*, 1998), and MRSA is a prevalent coloniser of the skin (Gordon & Lowy, 2008), stressing the importance of the colonised hospital environment and patients as a reservoir within the hospital allowing the opportunistic pathogens their opportunity. Also, cross-transmission from patient to patient, directly via lapses in infection control, or via contaminated instruments such as catheters (Dijkshoorn *et al*, 1987), is a fundamental problem in nosocomial infections.

In effect, the hospital environment can act as a training ground for bacterial populations. Bacteria are able to colonise the environment and patients, infecting those more susceptible, and during this colonisation they are likely to come into contact with many different kinds of antibiotic and other antibacterial agents. Only the strongest will survive; this leads to an increasingly resistant population being present in the hospital environment.

Antimicrobial resistance is a global problem, which has been likened to an independent disease entity. Of particular current concern, as highlighted by The 2005 Global Advisory on Antibiotic Resistance Data (GAARD) Report issued by the Alliance for the Prudent Use of Antibiotics (APUA) (APUA, 2005), is the rise of Gram-negative MDR bacteria, some of which are becoming untreatable, alongside exacerbation of the much-publicised MRSA problem with the increasing prevalence of community-associated MRSA (Section 1.2). Increasing travel and globalisation may be a factor in the worldwide spread of MDR strains, and there is the possibility that the global events of war and natural disasters may help the spread of resistant bacteria via global transfer of infected patients (McGowan Jr, 2006).

Initially Gram-positive bacteria were the major problem in the hospital environment, with clinicians struggling to control staphylococcal infections. The introduction and widespread use of antibiotics brought these infections under some control. This, however, appeared to allow Gram-negative bacteria to fill the vacant niche and they have become an increasing threat, typified by opportunistic infections in vulnerable

hospital patients and rapid development of resistance to many antimicrobials, probably helped by their prevalence in the environment, limiting the therapeutic options available (McGowan Jr, 2006). Currently, both Gram-positive and Gram-negative bacteria are causes for concern in the hospital environment, and examples of multi-drug resistance exist in both. Predominant amongst these are methicillin-resistant *Staphylococcus aureus* (Gram-positive) and *Acinetobacter baumannii* (Gram-negative). These two organisms are described in more detail in Sections 1.2 and 1.3.

Nosocomial (hospital-acquired) infections have been increasingly publicised in recent years following several outbreaks and celebrity 'endorsements' (TimesOnline, 2004). The use of sensational names such as 'superbug', commonly used to describe methicillin-resistant *Staphylococcus aureus* (MRSA) (Foster, 2004), has also helped to bring the problem of rising antibiotic resistance to the public's attention. Additionally, various initiatives and surveillance groups are now evident, such as the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) project, the National Nosocomial Infections Surveillance (NNIS) system in the US, the Antibiotics Resistance Prevention and Control (ARPAC) project and the aforementioned APUA and the GAARD project.

There is also greater acknowledgement of the problem in government as evidenced by initiatives such as the Scottish Executive Health Department Antimicrobial Resistance Strategy and Scottish Action Plan, introduced in 2002, which integrated the government

commitment to a UK Antimicrobial Resistance Strategy with an Action Plan for Scotland (Scottish Government website, 2002) and reports urging government action to tackle the problem such as those of the Infectious Diseases Society of American (IDSA) which led to the STAAR (Strategies to Address Antimicrobial Resistance) Act being presented to the US government in 2007 (IDSA website, 2007).

The outbreaks and prolonged patient colonisation that are associated with MDR bacterial infections not only draw negative publicity and increase the concern of individuals at the prospect of hospital stays but also have an economic cost. This includes costs connected with the specialised infection control practices needed to control an outbreak, such as barrier nursing, and disinfection of both wards and patients. A recent review of the direct health care cost of MRSA in Canada, for example, calculated an average cost of $12,216 per infected patient, with hospitalisation the main contributor to cost followed by barrier precautions, antimicrobial therapy and laboratory investigations (Goetghebeur *et al*, 2007). Likewise a study into the cost of hospital-acquired infections in a UK hospital estimated on average a cost for infected patients almost three times higher than that of uninfected patients, alongside a 2.5 times longer hospital stay, the largest contribution to additional costs being nursing care (Plowman *et al*, 2001).

Infection with MDR bacteria in the hospital can also be associated with increased morbidity and mortality as it can delay the administration of appropriate therapy and limit available therapeutic options. Whilst there can be debate about the attribution of mortality directly to MDR bacteria as they often infect those that already have a poor

prognosis, recent reviews have concluded that both *A. baumannii* and MRSA infections are associated with increased mortality (Falagas & Rafailidis, 2007; Falagas *et al*, 2006; Cosgrove *et al*, 2003; Whitby *et al*, 2001).

1.1.5 Dwindling Therapeutic Options

The situation of using toxic agents as a last resort treatment highlights a fundamental problem associated with the rise in antibiotic resistance; clinicians are rapidly running out of options to treat infections by MDR bacteria that have become resistant to all normal treatments. Dwindling therapeutic options are in part due to a lack of investment into the discovery and development of new antibiotic agents; the last decade has seen a steady decrease in the number of new antibiotics being approved. There has been much attention in recent years on the subject of why the big pharmaceutical companies have been investing less in this sector; it seems that the situation involves many factors such as a greater return on drugs for chronic conditions such as cardio-vascular disease, diabetes, arthritis, and from anti-HIV drugs, the need to prioritise in the light of the emergence of new global diseases, and an increase in safety regulations to be adhered to. These factors combine to give antibiotics a low placing on the list of priorities for companies (Spellberg *et al*, 2004; Projan, 2003). Rising costs of manufacture, pricing pressures and a higher safety profile requirement by the FDA means that many drugs in development do not reach the latter stages of the process (Spellberg *et al*, 2004). Indeed,

current commonly used agents which are highly active chemical compounds (such as ciprofloxacin) may not have been passed for use if subject to current regulations.

The tendency to derive new antibiotics from different iterations of older ones has also contributed to the current situation; great hope was placed upon new technologies to provide ways forward in drug discovery but so far these have not led to the multitude of new agents that was hoped for despite some progress in this area (Projan & Shlaes, 2004). Ironically, the very steps taken to slow the emergence of MDR bacteria may also have contributed to the current lack of new antibiotics in development; restricting the use of antibiotics is a fundamental measure in controlling the rise of resistance but of course, from the company's perspective, it leads to a reduction in market size and subsequently becomes a less attractive investment prospect (Projan & Shlaes, 2004).

1.1.6 Future Directions

It seems clear that the promise of the antibiotic era has ended. It is no longer hoped, at least not within the scientific community, that miracle cures can be found to rid the world of infectious diseases. Bacteria have a much longer history than humans and, rather than trying to defeat them, focus is needed on preserving the usefulness of current treatments and attempting to slow the trend of rising antibiotic resistance; calling a truce instead of aiming to win a war. Reversal of the resistance process, whilst it may be

possible through replacement with susceptible strains (Levy & Marshall, 2004), seems an increasingly naïve proposition (Livermore, 2007).

Initial steps towards halting the rise of resistance must include improved surveillance with faster and more reliable typing methods to further delineate which strains are important in the epidemiology of hospital infections (Levy & Marshall, 2004; APUA, 2005). As mentioned there are several groups that are involved in monitoring the global spread of resistant bacteria, including SCOPE, NNIS and ARPAC, and government supported initiatives and strategies to tackle the problem. It is important that surveillance measures attempt to examine a spectrum of isolates to get a true picture of the epidemiological aspects, rather than purely reactive surveillance in response to outbreaks (Dijkshoorn *et al*, 2007), though of course there is value in specifically studying outbreak strains.

Infection control measures are vital to help prevent transmission and persistence of bacteria in general and MDR bacteria specifically within the hospital environment and larger community. The plethora of active antibiotics led to leniency in infection control procedures in the past; however it now seems that healthcare professionals are once again very aware of how important infection control measures such as reduced patient contact and hand-washing can be, and there is evidence for such measures successfully controlling outbreaks.

A recent study demonstrated successful control of an outbreak of carbapenem-resistant *A. baumannii* in the neurosciences critical care unit of a UK hospital by early management and patient segregation, screening of patients and the environment (which was found to be heavily contaminated), and subsequent increased environmental cleaning and hand hygiene (Enoch *et al*, 2008). Equally, increased infection control and hand hygiene have been shown to have successfully reduced the occurrence of MRSA infections (Grayson *et al*, 2008). However, with the efficacy of infection control comes the increased used of disinfectants and antiseptics, which are becoming prevalent not only in the clinical environment, but also in the community at large, even being found in make-up and toothpaste (Levy, 2001). Biocides and their possible implications for antimicrobial resistance in MRSA are discussed in more detail in Section 1.2.

A more rational and controlled use of antibiotics is required to reduce the selective pressure that they provide. However clinicians are often under pressure, sometimes from the patients themselves, to prescribe antibiotics, and sometimes the information provided to clinicians to guide their prescribing is confusing (Fleming, 2007). This situation has improved somewhat with campaigns to highlight the problems of antibiotic resistance and improved public awareness. The media in this respect has been immensely helpful in bringing the issue of MDR nosocomial pathogens into the public eye. However their coverage often borders on the hysterical and could lead to misinformation. Continual non-sensational education is important to maintain and increase public awareness of the issues, and encourage both patients and clinicians against the misuse of antibiotics.

Improved cooperation between industry, academia and government, maybe even with incentives being offered to encourage research into new antibiotic possibilities, may be needed to encourage the initiation of more antibiotic discovery and development programmes. Different approaches rather than modification of old compounds would also be useful in finding new agents to combat infections; any new products may have limited usefulness as it seems likely that bacteria will continue to adapt to their environment and develop resistance to whatever new compounds are used against them, but those which are entirely novel would have the advantage, even in the short term, over new iterations of old formats. However, given the lack of either iterations or novel antibiotics in development, immediate priorities must focus on other means of slowing the development of resistance (Projan & Shlaes, 2004; Spellberg *et al*, 2004).

The best way to gain some control over nosocomial infections is to increase our understanding of bacterial resistance development; not only is this essential to pinpoint new targets for antibiotic agents, it is also vital to enable the implementation of effective methods to slow the seemingly relentless rise of resistance, and to forewarn against potential new aspects of resistance development.

1.2 MRSA and the Increased Use of Biocides

1.2.1 Gram-positive Bacteria

Gram positive bacteria have historically been important pathogens in the hospital environment and they continue to present clinicians with major treatment and infection control problems. For example, analysis of data collected for the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) project reported that 65% of the nosocomial bloodstream infection cases reported from 49 US hospitals over 7 years were due to Gram-positive organisms, and of these 20% were *Staphylococcus aureus* (Wisplinghoff *et al*, 2004).

Staphylococci are common skin commensals and have long been problematic in hospitals, causing urinary and respiratory tract infections and bacteraemia. They are also common infective agents during surgery due to their commensal nature; numerous sites on the body can be colonised and this provides a reservoir and important routes of infection when the host defences are breached (Gordon & Lowy, 2008). Staphylococcal infections were tackled at first with penicillin, at the advent of the antibiotic era, but developed resistance first to penicillin and then to further antibiotics developed against them, including methicillin.

1.2.2 MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) has received the greatest publicity of the nosocomial MDR bacteria, as evidenced by its frequent appearance in the media, often termed 'superbug' (Foster, 2004). Methicillin was introduced in 1959 in Europe to treat infections by penicillin-resistant *S. aureus*, as the rates of resistance to penicillin were already high. It was initially successful but resistant strains developed, following a pattern similar to earlier penicillin resistance development. The first MRSA strain was reported in the UK in 1961, and others were subsequently reported worldwide. Several outbreaks of MRSA followed in the 1970s, usually associated with high methicillin use in ICUs and, during the 1980s, MRSA became a significant problem globally (Rice, 2006; Boucher & Corey, 2008),

Resistance to methicillin is via the expression of the *mecA* gene, encoding the low-affinity penicillin binding protein (PBP) 2a which also confers resistance to other β-lactams. *mec*A is carried on a mobile genetic element called the staphylococcal chromosomal cassette (SCC)*mec*, of which there are several types; Types I, II and III are normally associated with HA-MRSA and Types IV and V with community-associated (CA)-MRSA (Hiramatsu *et al*, 2001; Otter & French, 2006; Stryjewski & Chambers, 2008).

1.2.3 Impact of MRSA

The term MRSA is a useful label but in fact hospital-acquired (HA) MRSA is generally multi-drug resistant and demonstrates a clonal pattern of spread (Amyes, 2000). For example the two epidemic MRSA (EMRSA) clones EMRSA-15 and EMRSA-16 are predominant in the UK, and are highly resistant to most antibiotics (Amyes 2005). MRSA can colonise the skin and particularly the anterior nares of a patient, transiently or persistently. MRSA has also been found to persist in the hospital environment on inanimate objects and in the area surrounding patients. Transmission is thought to be mainly via infection control lapses by healthcare practitioners, with patients and the hospital environment as a reservoir. In addition, patient-to-patient and visitor-to-patient contact may be important transmission routes of bacteria within the hospital setting (Gordon & Lowy, 2008; Gould, 2005).

As mentioned, MRSA is highly associated with nosocomial bloodstream infections (Wisplinghoff *et al*, 2004) and can also be implicated in surgical-site infections, pneumonia, endocarditis, osteomyelitis and invasive skin infections (Gordon & Lowy, 2008). The percentage of MRSA bloodstream infections in the US was found to be significantly higher in the ICU where the most vulnerable patients are found, and the percentage of *S. aureus* bloodstream infections that were MRSA had risen from 22% to 57% from 1995 to 2001 (Wisplinghoff *et al*, 2004). There is evidence that MRSA bloodstream infections may be levelling off in certain areas; for example, Health Protection Agency (HPA) figures show a decline in cases reported in England from

April 2006 to September 2007 (HPA website, 2008a). However this may be partially due to changes in the surveillance system, and rates are currently still high compared to other European countries (Gould, 2005).

Although, as with *Acinetobacter* spp (Section 1.3.3), attributable mortality directly due to MRSA can be unclear due to the association with patients with adverse prognosis, several meta-analyses comparing MRSA to methicillin-susceptible *S. aureus* (MSSA) have indicated MRSA infection is associated with greater mortality, morbidity and treatment costs (Cosgrove *et al*, 2003; Whitby *et al*, 2001). Whilst their conclusions regarding MRSA versus MSSA can be questionable due to broad inclusion criteria and possible lack of accounting for all factors, they do indicate an association between MRSA infection and increased mortality. Additionally, it is apparent that difficulties in the treatment of MRSA will also contribute to increased mortality. MRSA outbreaks are also associated with high economic costs; this includes antimicrobial therapy, care of MRSA-infected patients, infection control procedures and surveillance (Gould, 2005). As mentioned, a recent review of the direct health care cost of MRSA in Canada calculated an average cost of $12,216 per infected patient, with hospitalisation the main contributor to cost, followed by barrier precautions, antimicrobial therapy and laboratory investigations (Goetghebeur *et al*, 2007).

1.2.4 Community-associated MRSA

Initially MRSA was solely a hospital problem, but it is now apparent that it can also be community associated or acquired (CA-MRSA), causing infections in patients with no previous history of healthcare contact. This is highlighted by the recent publication of guidelines by a Working Party on behalf of the British Society for Antimicrobial Chemotherapy (BSAC) for the diagnosis and management of MRSA infections presenting in the community (Nathwani *et al*, 2008), which aim to increase awareness of the problem of CA-MRSA in the hopes of preventing and controlling recurrence and exacerbation of the problems seen with HA-MRSA.

CA-MRSA commonly causes skin and soft tissue infections, though it may also cause more serious infections such as necrotising pneumonia. There are several differences between CA-MRSA and HA-MRSA; CA-MRSA is capable of rapid spread and causes a higher rate of infection than HA-MRSA, but tends not to be multi-drug resistant and is polyclonal (Stryjewski & Chambers, 2008; Nathwani *et al*, 2008). Additionally the presence of the smaller SCC*mec* Types IV and V in CA-MRSA as opposed to Types I, II and III in HA-MRSA is currently a useful molecular distinction (Otter & French, 2006; Stryjewski & Chambers, 2008), although ciprofloxacin susceptibility has also been reported as a helpful marker for CA-MRSA strains identified in the London area (Otter & French, 2008a).

This study, reporting the recent emergence of CA-MRSA in a London hospital (Otter & French, 2008a), also highlights a growing concern arising from CA-MRSA; its increasing movement into hospitals and associated outbreaks. This is a particularly disconcerting aspect, as the movement of these strains between hospital and community could lead to a greater number at risk of infection in both environments, and the development of multi-drug resistance in CA-MRSA due to antibiotic pressure present in the hospital environment (Otter & French, 2006).

The recent epidemiological advance of MRSA in the form of CA-MRSA is hence a growing area of concern, not only for its impact in the community, but also in the clinical environment, furthering the urgency of the need to control MRSA infection and spread.

1.2.5 Control of MRSA

Glycopeptides, including vancomycin, are currently used to treat serious MRSA infections. However, there are reports of vancomycin intermediate and vancomycin resistant *S. aureus* (VISA and VRSA respectively) which are of growing concern for treatment options (Wang *et al*, 2006). Daptomycin, a cyclic lipopeptide recently approved for use in certain infections, has shown rapid bactericidal activity against *S. aureus*, including against resistant strains (French, 2006). However, further trials are needed to confirm the usefulness of this agent against MRSA in the clinical environment.

In light of the reduction in treatment options, it is vital in the meantime to employ suitable infection control measures to: minimise the spread of MRSA in hospitals, reduce the emergence of resistant isolates, and reduce the movement of CA-MRSA into and within the hospital environment. Given the aforementioned colonisation and transmission of HA-MRSA within hospitals, good infection control procedures would seem to be of great importance.

Indeed, samples of worldwide national guidelines stress the importance of early detection through surveillance, isolation, decolonisation and hand hygiene, with increasingly evidence based approaches for guidelines (Humphreys, 2007). Recently a report on the outcome of a multi-site hand hygiene culture-change programme in Australian hospitals indicated a significant reduction in both the number of patients with MRSA bacteraemia and the number of clinical MRSA isolates, as measured per 100 patient discharges per month and compared to mean baseline rates (Grayson *et al*, 2008). Whilst one of the products used was developed by the authors, its composition was similar to the other product used (0.5% chlorhexidine with 70% ethanol or 70% isopropanol respectively) so this is unlikely to have biased the results. There are novel methods of cleaning being developed with success, such as the recently reported inactivation of bacteria renowned for survival in the hospital environment (MRSA, *Acinetobacter* spp, *Clostridium difficile* and *Klebsiella pneumoniae*) with hydrogen peroxide vapour (Otter & French, 2008b), but these are not yet regularly used. As such infection control methods currently involve the use of biocides.

1.2.6 Rise of Biocide Use

With the re-establishment of the importance of infection control, there has been a concurrent rise in the use of biocides. Biocides are used as antiseptics, disinfectants, preservatives and some, such as chlorhexidine, are used for all three purposes. They are frequently used in the hospital environment for disinfection of inanimate objects, as antiseptics or topical antimicrobials on the skin, and as preservatives in products to avoid contamination (Russell, 2003), with suggested incorporation into invasive equipment such as endotracheal tubes a recent manifestation (Pacheco-Fowler *et al*, 2004). Hence, biocides are aimed at prevention and control, whereas antibiotics are for the treatment of infections. But whilst they are of increasing importance and usefulness in the hospital environment, there has also been a rise in biocides incorporated into a wide range of products within healthy households; for example in toothbrushes, soaps, cleansers, food storage containers and even chopsticks (Levy, 2001).

1.2.7 Chlorhexidine

Chlorhexidine is an antiseptic solution that has been used since the 1950s for applications including hand-washing, treatment of gingivitis and preoperative skin preparation. It is a cationic biguanide which, at high concentrations, binds to the bacterial cell wall, altering the osmotic equilibrium and causing cell death via precipitation of the cytoplasmic contents. At low concentrations it affects membrane integrity. There is evidence supporting its safety in a wide variety of clinical uses

(Milstone *et al*, 2008) and it is also a frequent ingredient in mouthwashes and dental products in the home.

1.2.8 Reduced Susceptibility to Biocides

The rise in biocide usage has been accompanied with concerns that their overuse and misuse could lead to the emergence of bacteria with reduced susceptibility to biocides, and even cross-resistance to antibiotics (Russell *et al*, 1998; Levy, 2001). Despite the increase in biocide products, there has not been a simultaneous increase in our understanding of the interaction of biocides with bacteria (Maillard, 2007).

Possible worse-case scenario implications of biocide reduced susceptibility in the hospital environment could include failure of preoperative, hand hygiene and environmental disinfectants and antiseptics, leading to the possibility of increased auto-, cross- and environmental infection rates respectively. This could also have implications upon antibiotic usage by increasing the need for them to treat infections (Cookson, 2005).

There are several problems associated with the subject of biocide reduced susceptibility. Compared to antibiotic resistance there is a low profile and little funding into research, with no structured surveillance systems. Additionally there are currently no internationally agreed efficacy tests or tests for biocide reduced susceptibility (Cookson, 2005). The European suspension test has been developed but may not adequately

address the clinical situation; for example the presence of bacterial biofilms and biocide residues in the hospital environment. Thomas and colleagues have developed some promising methods to test not only bacteria susceptibility to biocides in suspension, but also to biocide residues and biocides' efficacy upon dried surface bacteria (Thomas *et al*, 2005). Another issue is the lack of evidence at present to support reduced susceptibility to biocides in practice, and difficulties in correlating *in vitro* experiments with the practical clinical situation (Maillard, 2007).

Whilst there are some similarities in specific antibiotic and biocide mechanisms of action, there is a fundamental difference; whereas many antibiotics exert their effect by growth inhibition via a specific target, biocides usually have multiple targets which are concentration-dependent, their desired rapid kill effect apparent at in-use concentrations, with subtle effects occurring at low concentrations (Russell, 2003). As such, the terminology in relation to biocides is to use 'reduced susceptibility' as opposed to 'resistance', and MICs are not generally considered a suitable means of testing biocide reduced susceptibility (Bloomfield, 2002).

Biocide efficacy is generally dependant on rapid kill effects, which can be measured by the microbiocidal effect (ME): the log reduction in cell number compared to a control (Thomas *et al*, 2005). There is a suggestion that efficacy testing of biocides should use an equal to or greater than a 5 log reduction (equivalent to 99.999%) in numbers of challenged organisms after contact as the boundary for an acceptable ME (Payne *et al*, 1999).

1.2.9 Mechanisms of Reduced Susceptibility to Biocides

Biocide reduced susceptibility is not a new occurrence and has been reported in laboratory studies (Thomas *et al*, 2000; Kõljalg *et al*, 2002; Moken *et al*, 1997; Kampf *et al*, 1998). However, reports from the clinical environment are rare as it can be difficult to correlate biocide reduced susceptibility with the clinical situation, and there is a lack of awareness of the potential problem (Maillard, 2007). Suggested resistance mechanisms are defined, similarly to antibiotic resistance (Sections 1.1.2 and 1.5.1), as both intrinsic and acquired. Mechanisms of resistance to biocides include reduced uptake by impermeability (via porins, the outer membrane, cell envelope and biofilm formation) or by active efflux, and modification (via target modification or overproduction of targets) (Maillard, 2007; Russell, 2003).

There is debate about whether laboratory studies of biocides relate well to the clinical situation as the use of biocides in the clinical environment is generally at very high concentrations; however, the study of biocide reduced susceptibility at low concentrations is increasingly being advocated (Maillard, 2007; Russell, 2003). This is partly because of the increasing incorporation of low concentrations of biocides into products (surfaces, liquids and textiles) throughout the domestic and more importantly clinical environments (Levy, 2001). Also the use of some biocides (such as chlorhexidine) as preservatives in solutions, cosmetics and other domestic items alongside their simultaneous use for de-contamination leads to the concern that reduced

susceptibility could result from these low concentrations and lead to their use for disinfection and antisepsis being compromised (Maillard, 2007).

Additionally it is likely that even in clinical use low concentrations of biocide will be present at some point via misuse (for example the topping-up of biocide containers or failure to remove residue) and excessive use (for example general cleaning and disinfection rather than targeted use); indeed, it has been suggested that there is likely to be a continuum of biocide concentration in the environments in which they are used, and as such sub-lethal concentrations of biocide for their targets could be present, meaning that low concentrations of biocide would act more akin to antibiotics and specifically affect only certain targets (Bloomfield, 2002).

Exposure to low concentrations of chlorhexidine has been reported to lead to selection of reduced susceptibility isolates. Stable reduced susceptibility in *P. aeruginosa* developed following exposure to increasing concentrations of chlorhexidine, and also after repeated exposure to a residual concentration of chlorhexidine (Thomas *et al*, 2000). Additionally some tested antibiotic resistant strains were found to have less susceptibility to chlorhexidine, suggesting that selective pressure from the biocide could select out antibiotic resistant strains (Kõljalg *et al*, 2002).

Cross-resistance to antibiotics is a particularly disconcerting aspect of this area, and there are several additional reports of parallel resistance to antibiotics. For example *E. coli* mutants selected for reduced susceptibility to pine oil also showed resistance to

several antibiotics and overexpression of the *marA* gene, which activates the multidrug AcrAB efflux pump, indicating that biocides were acting in the same way as antibiotics (Moken *et al*, 1997; Levy, 2002b). It does seem that a situation where use of low concentrations of biocides leading to reduced susceptibility and also to parallel antibiotic resistance is not unlikely and warrants further investigation, especially as there is a lack of information about clinical isolates (Russell *et al*, 1998).

Of further concern is that the effectiveness of biocides can vary with different strains, as highlighted in a study where MRSA isolates were reported to be significantly less susceptible to chlorhexidine than MSSA isolates (Kampf *et al*, 1998). Also, in an evaluation of the standard strains used to test biocide efficacy against clinical isolates it was found that with a shorter contact time microbiocidal effects were higher for both *P. aeruginosa* and *S. aureus* ATCC strains than the tested clinical strain for these organisms (Payne *et al*, 1999). This questions the use of standard strains in biocide efficacy testing, and whether they are sufficient to test the effectiveness of biocides against current clinical isolates.

As such, it is important to study the effects of biocides at low concentrations, on clinical isolates compared to standard strains. Given the renewed importance of infection control in combating the spread of MRSA and the increased use of biocides, early warning as to reduced susceptibility of these prevalent nosocomial pathogens is vital.

1.3 *Acinetobacter*

Gram-negative non-fermenters, those unable to ferment glucose as an energy source, have become an increasing concern as hospital pathogens in recent years. It is thought that Gram-negative bacteria began to predominate in the niche created by a reduction of Gram-positive bacteria in the hospital environment during the heyday of antibiotics. Gram-negative non-fermenters are opportunistic pathogens, prevalent in the hospital environment as colonisers and predominantly causing infections in those who are seriously ill or immunocompromised (Falagas & Bliziotis, 2007).

There is much attention focused on the rise of Gram-negative bacteria but the fundamental problem is not so much an increase in infections caused as the growing prevalence of strains that are multi-drug resistant and in some cases pan-drug resistant (Kuo *et al*, 2004). A recent review of nosocomial Gram-negative infections, using data from the National Nosocomial Infections Surveillance (NNIS) System, concluded that the percentages of Gram-negative bacteria associated with the top four nosocomial infections (pneumonia, surgical site infection, urinary tract infection and bloodstream infection) had not significantly changed from 1986 to 2003, with the exception of ICU pneumonia episodes associated with *Acinetobacter* which had increased. However, significant increases in resistance levels were observed and it is this aspect which is of greatest concern clinically (Gaynes *et al*, 2005).

1.3.1 The *Acinetobacter* genus

Acinetobacter spp are a prime example of this group of bacteria. In the last 20 years the *Acinetobacter* genus has emerged as a problem pathogen in hospitals worldwide, implicated in nosocomial infections including bacteraemia, secondary meningitis and pneumonia, and has generated much attention in the scientific community due to its ability to rapidly acquire resistance (Perez, 2007). It is an opportunistic pathogen, considered generally harmless to colonised healthy individuals but often leading to increased morbidity and mortality when infecting immuno-compromised patients (Falagas & Rafailidis, 2007). As well as the ability to rapidly acquire multi-drug resistance *Acinetobacter* spp have the capacity for long-term survival in the hospital environment (Jawad *et al*, 1998; Das *et al*, 2002). As such hospital ICUs and the patients within provide a niche for *Acinetobacter*, with an environment in which they may be exposed to many different antibiotics and have the opportunity to accumulate resistance. The more recent growing prevalence of carbapenem-resistant (Evans *et al*, 2008) and pandrug-resistant (Kuo *et al*, 2004) isolates of *Acinetobacter baumannii* is of particular concern to clinicians, threatening their ability to treat infections.

Acinetobacter spp comprise Gram-negative coccobacilli which are strictly aerobic, nonmotile, catalase positive and oxidase negative (Bergogne-Bérézin & Towner, 1996). The *Acinetobacter* genus is genotypically heterogenous and has a complex and confusing taxonomic history which has been refined in the last 20 years in tandem with the emergence of *Acinetobacter* spp as nosocomial pathogens (Dijkshoorn *et al*, 2007).

A phenotypic classification system for *Acinetobacter* speciation was first described in 1986 (Bouvet & Grimond, 1986) which, together with subsequent systems (Gerner-Smidt *et al*, 1991), was useful for identifying most but not all *Acinetobacter* spp. DNA-DNA hybridisation has been used to further delineate species (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989) and there are currently 18 named *Acinetobacter* spp and a further 14 genomic species (gen.sp.), encompassing both clinical and environmental strains (see Table 1.2).

The key aspects of this genus are the prevalence of *Acinetobacter* spp in hospitals, their association with nosocomial infections and, most importantly, their ability to rapidly develop multi-drug resistance. *Acinetobacter baumannii* is regarded as the most important clinical species of the genus, most frequently involved in nosocomial infections (Bouvet & Grimond, 1986; Bergogne-Bérézin & Towner, 1996; Wisplinghoff *et al*, 2000). After *A. baumannii*, genospecies (gen.sp.) 3 and gen.sp. 13TU are the most prevalent species in clinical specimens (Dijkshoorn *et al*, 2007), and there is evidence that these species may also be implicated in nosocomial infections.

Acinetobacter gen.sp.3 has been associated with tracheobronchitis infection (Dijkshoorn *et al*, 1993) and *Acinetobacter* gen.sp. 13TU is particularly evident in the hospital environment; for example, gen.sp.13TU was responsible for hospital outbreaks in the Netherlands (van Dessel *et al*, 2002; van den Broek *et al*, 2006), has been reported to be prevalent in Korean hospitals with a greater resistance rate to imipenem than *A.baumannii* isolates (Lee *et al*, 2007) and, with *A. baumannii*, appears to be a prevalent

cause of infections in UK hospitals (Spence *et al*, 2002). The increasing reports of gen.sp.13TU may be partly due to an increased awareness of its genetic similarity to *A. baumannii* and the growing ability to discriminate between the two, as mentioned below in Section 1.3.2.

Table 1.2 – Species and genomic species of the *Acinetobacter* genus
(adapted from Dijkshoorn *et al*, 2007)

Species name	Source
Acinetobacter calcoaceticus (species 1)	Soil and humans (inc. clinical)
Acinetobacter baumannii (species 2)	Humans (inc. clinical), soil, meat, vegetables
Gen.sp. 3	Humans (inc. clinical), soil, vegetables
Acinetobacter haemolyticus (species 4)	Humans (inc. clinical)
Acinetobacter junii (species 5)	Humans (inc. clinical)
Gen.sp. 6	Humans (inc. clinical)
Acinetobacter johnsonii (species 7)	Humans (inc. clinical) and animals
Acinetobacter lwoffii (species 8 & inc. gen. sp. 9)	Humans (inc. clinical) and animals
Gen.sp. 10	Humans (inc. clinical), soil, vegetables
Gen.sp. 11	Humans (inc. clinical) and animals
Acinetobacter radioresistens (species 12)	Humans (inc. clinical), soil and cotton
Gen.sp. 13BJ or 14TU	Humans (inc. clinical)
Gen.sp. 14BJ	Humans (inc. clinical)
Gen.sp. 15BJ	Humans (inc. clinical)
Gen.sp. 16	Humans (inc. clinical) and vegetables
Gen.sp. 17	Humans (inc. clinical) and soil
Gen.sp. 13TU	Humans (inc. clinical)
Gen.sp. 15TU	Humans (inc. clinical)
Gen.sp. ‘between 1 and 3’	Humans (inc. clinical)
Gen.sp. ‘close to 13TU’	Humans (inc. clinical)
Acinetobacter ursingii	Humans (inc. clinical)
Acinetobacter schindleri	Humans (inc. clinical)
Acinetobacter parvus	Humans (inc. clinical)
Acinetobacter baylyi	Activated sludge and soil
Acinetobacter bouvetii	Activated sludge
Acinetobacter towneri	Activated sludge
Acinetobacter tandoii	Activated sludge
Acinetobacter grimontii	Activated sludge
Acinetobacter tjernbergiae	Activated sludge
Acinetobacter gerneri	Activated sludge
Acinetobacter venetianus	Sea water

Letters indicate the initials of the authors of the studies in which the species were designated (TU = Tjernberg & Ursing, 1989. BJ = Bouvet & Jeanjean, 1989).

1.3.2 Typing and Speciation

Detailed surveillance of isolates both within and between hospitals, and in the worldwide community, is essential for understanding the epidemiology of organisms, especially during outbreaks where knowing whether the infecting isolates are clones or endemic to the patient may have impacts on both eradication and treatment options.

PFGE with restriction by *Apa*I is considered a reliable and reproducible method for typing of *Acinetobacter* isolates (Gouby *et al,* 1992; Seifert *et al,* 1994). Use of the advocated method (Bannerman *et al,* 1995) with the control strain RUH 2034 for gel normalisation, allows comparison of resulting band patterns between laboratories via the Antibiotic Resistance Prevention and Control (ARPAC) project's database (Towner *et al,* 2008).

The species most frequently involved in nosocomial infections, *A. baumannii*, can not be easily separated from *A. calcoaceticus* (a predominantly environmental species), gen.sp. 3 or gen.sp. 13TU (also clinically relevant species) by phenotypic means. Commercial systems such as the automated Vitek™ system and the API 20NE system from bioMériux™, commonly used for identification in diagnostic laboratories, are generally considered unsuccessful at delineating different *Acinetobacter* spp and often classify various species as *A. baumannii* (Bernards *et al,* 1996; Apisarnthanarak *et al*, 2007; Zbinden *et al,* 2007). Even DNA-DNA hybridisation, which remains the 'gold standard' for determining whether organisms belong to the same species but is outwith the

capabilities of most laboratories to perform, does not distinguish well between these species, particularly between *A. baumannii* and gen.sp. 13TU (Tjernberg & Ursing, 1989). It has been proposed that these 4 species be grouped together into the *A. calcoaceticus-A. baumannii* (Acb) complex (Gerner-Smidt *et al,* 1991). However, this may not be considered wise from a clinical standpoint as it groups the environmental strain *A. calcoaceticus* with the prevalent clinical strains (Dijkshoorn et al, 2007).

The confused taxonomy of the *Acinetobacter* genus has meant that a quick, reliable and reproducible speciation technique which can be employed throughout clinical and research laboratories has often been lacking, resulting in confusion and frequent misidentification of other species as *A. baumannii*. As such, several genotypic procedures have been developed for the identification of all *Acinetobacter* spp. including tRNA spacer (tDNA) fingerprinting, amplified fragment length polymorphism (AFLP), amplified 16S ribosomal DNA restriction analysis (ARDRA) and DNA sequence analysis (Ehrenstein *et al,* 1996; Vaneechoutte *et al,* 1995; Janssen *et al,* 1997; Chang *et al,* 2005).

The tDNA fingerprinting method, which amplifies the region between tRNA genes (Welsh & McClelland, 1991), is a valuable tool for the rapid identification of most *Acinetobacter* spp (Ehrenstein et al, 1996; Spence *et al,* 2002; Higgins, 2002), although it does not successfully distinguish between *A. baumannii* and gen.sp. 13TU.

Recently there is increasing evidence that *A. baumannii* has a naturally occurring carbapenemase gene, which appears to be intrinsic to this species but absent from other *Acinetobacter* spp. The *bla*$_{\text{OXA-51}}$ gene was first described by Dr Susan Brown and colleagues (Brown *et al,* 2005) and since then several highly-related variants have been described in global isolates, and are referred to as *bla*$_{\text{OXA-51-like}}$ genes (Turton et al, 2006; Evans *et al,* 2008). It is a matter of some debate whether or not they are present in all isolates of the species, but a study screening a variety of well-characterised isolates including *Acinetobacter* spp, *Acinetobacter* type strains and other Gram-negative organisms concluded that only isolates of the *A. baumannii* species gave a positive result for the presence of *bla*$_{\text{OXA-51-like}}$ genes (Turton et al, 2006). This included the ATCC 19606 *A. baumannii* type strain. At present it seems reasonable to consider identification of the *bla*$_{\text{OXA-51-like}}$ genes a tool for detecting *A. baumannii,* and to enable distinction between *A. baumannii* and gen.sp. 13TU.

Analysis of the 16S-23S rRNA intergenic spacer sequence, which takes advantage of the low intraspecies variation but high interspecies diversification of these regions, has also been shown to be a valuable tool for distinguishing species of the Acb complex (Chang *et al*, 2005).

1.3.3 Clinical Impact

Acinetobacter are often mistakenly considered to be ubiquitous in nature, however this does not apply to all species of the genus. The natural reservoir of *A. baumannii* and

other species isolated in the clinical environment is unclear, but it is thought that the main transmission routes are via fluid and instrument contamination, from colonised patients and health-workers in hospital, and via direct contact (Dijkshoorn *et al*, 2007). Whilst *Acinetobacter* spp may be found in other wards, ICU wards and patients are most frequently affected by *A. baumannii* (Cisneros & Rodríguez-Baño, 2002), with risk factors for infection including immuno-suppression, invasive procedures and previous antimicrobial therapy (García-Garmendia *et al*, 2001).

It has been reported that *Acinetobacter* spp can spread via airborne transmission within hospitals (Bernards *et al*, 1998) and transmission can also be via cross-infection between patients (Dijkshoorn *et al*, 1987). Additionally *Acinetobacter* spp are able to survive for long periods on dry surfaces (Jawad *et al*, 1998) and have been found to colonise surfaces and fabrics in the hospital environment, including equipment, bed linen and curtains (Das *et al*, 2002), highlighting the importance of the environment as a reservoir. There is even a suggestion that vegetables in particular may provide a route of introduction into the hospital environment (Berlau *et al*, 1999), although it is unclear whether *Acinetobacter* were present as colonisers or as a result of environmental contamination.

Whilst *A. baumannii*, and to a lesser extent gen.sp. 13TU, are the most frequently associated with hospital infections, further *Acinetobacter* species are also isolated from the clinical environment. These are often considered contaminants when associated with clinical samples, but there is some evidence of their involvement in infections, for

example *A. johnsonnii* has been associated with catheter-related bloodstream infection (Seifert *et al,* 1993). It is likely that generally these other species are colonisers; in studies examining carriage of *Acinetobacter* spp, *A. lwoffii, A. johnsonii, A. radioresistens* and gen.sp. 3 were frequently isolated from healthy human skin, whilst *A. baumannii* and gen.sp. 13TU were only rarely isolated (Seifert *et al*, 1997; Berlau *et al*, 1999). Although *A. baumannii* appears to be the species of greatest clinical importance, further investigation has been advocated if there is repeated isolation of other species (especially gen.sp. 13TU) associated with clinical symptoms (Bergogne-Bérézin & Towner, 1996) and it may be that these strains have as yet undiscovered importance in the clinical environment.

The clinical impact of *Acinetobacter* infection is a matter of debate (Dijkshoorn *et al*, 2007); as infections frequently occur in patients with an underlying disease, it has been argued that associated mortality is not a direct result of *Acinetobacter*. However, a recent analysis has concluded that *A. baumannii* infections are associated with increased mortality (Falagas & Rafailidis, 2007). In the hospital environment *Acinetobacter* infections are most frequently associated with pneuomonia, especially ventilator-associated pneumonia, and bloodstream infections (Joly-Guillou, 2005). Increased *Acinetobacter*-associated pneumonia has been reported in a study analysing Gram-negative bacteria associated with the most common nosocomial infections in the US (Gaynes *et al*, 2005). Additionally there are increasing reports of *Acinetobacter* infections occurring in situations out with the hospital setting. These include community-acquired infections, for example community acquired pneumonia associated

with *A. baumannii* (Anstey *et al*, 2002), and infections in trauma patients, for example a prevalence of *Acinetobacter* wound infections reported during military operations (Davis *et al*, 2005).

1.3.4 Epidemiology

There are global reports of multi-drug resistant *A. baumannii* in hospitals, often causing outbreaks (Perez *et al*, 2007). There is also evidence of the spread of multi-drug resistant *A. baumannii* strains between geographically related hospitals; in the UK circulation of several widespread outbreak strains of *A. baumannii* has been reported, the most prevalent in the UK being the OXA-23 clone 1 (Turton *et al*, 2004; Coehlo *et al*, 2004), and inter-hospital spread has been reported in other countries including the Netherlands (van den Broek *et al*, 2006) and the USA (Quale *et al*, 2003). Additionally, clonal spread is evident both within hospitals (Wisplinghoff *et al*, 2000) and between hospitals; for example clones I, II and III (Dijkshoorn *et al*, 1996; van Dessel *et al*, 2004) are widespread in Europe (Van Looveren & Goossens, 2004). Their widespread occurrence may be due to their presence in the community, and expansion in hospitals under antibiotic selective pressure (Dijkshoorn *et al*, 2007). A recent study of isolates from 25 hospitals in 17 European countries has suggested that broader lineages of carbapenem-resistant *A. baumannii* are also circulating through European hospitals (Towner *et al*, 2008).

The spread of carbapenem-resistant *Acinetobacter* is of particular concern, as these agents are often one of the only treatment options available (Evans *et al,* 2008). Recent Health Protection Agency (HPA) figures, examining data voluntarily reported to the HPA for *Acinetobacter* spp bacteraemia in England, Wales and Northern Ireland from 2003 to 2007, shows a significant increase in resistance to carbapenems (Imipenem and Meropenem) over this period, from 7% to 24% and 3% to 22% respectively (HPA website, 2008b). Most worrying is increasing reports of pandrug-resistant isolates (Kuo *et al,* 2004), although the term pandrug must be used cautiously as in fact the isolates may still be susceptible to rarely-used agents, such as polymyxin.

1.3.5 Antibiotic Resistance Mechanisms of *Acinetobacter* spp.

Acinetobacter was traditionally considered an organism with low pathogenicity, and so there were few studies into virulence factors. However, the recognition of the potential of *A. baumannii* in particular to cause specific mortality associated with infections has led to increased research into virulence factors, though this is still at a relatively early stage. Factors that are important for survival may include the ability to survive on dry surfaces (Jawad *et al,* 1998), metabolic versatility (Bouvet & Grimont, 1986), resistance to disinfection (Wisplinghoff *et al,* 2007), biofilm formation (Tomaras *et al,* 2003) and of course antibiotic resistance. The latter is undoubtedly the most important aspect of nosocomial *Acinetobacter*, particularly of *A. baumannii;* the ability to rapidly acquire multi-drug resistance, when combined with prolonged survival in the nosocomial

environment, is the main factor that renders *A. baumannii* a problematic opportunistic pathogen.

Specific resistance mechanisms of *A. baumannii* for the different antibiotic classes are in common with other bacteria and are generally well known. Some of the mechanisms which are found in *A. baumannii* are summarised in Table 1.3, and include target site alteration to prevent drug action, enzymatic modification of the drug and efflux systems that reduce the accumulation of drug in the bacterial cell.

Table 1.3 – Mechanisms of resistance in *Acinetobacter baumannii*
Adapted from Dijkshoorn *et al*, 2007

Class of antimicrobial	**Mechanism of resistance**	**Examples of components**	
Quinolones	Target alteration	GyrA ParC	Detailed in Section 1.4.
Carbapenems	β-lactam hydrolysis	OXA-51-like	Chromosomally encoded Class D β-lactamases Thought to be intrinsic to *A. baumannii*
		OXA-23-like	Plasmid-encoded Class D β-lactamases
		IMP-1 and others VIM-2, SIM-1	Class B metallo-β-lactamases
	Changes in OMPs	CarO	OMP implicated in drug influx
Aminoglycosides	Aminoglycoside modification	e.g. AAC(3)-Ia	Acetyltransferases
		e.g. APH(3')-Ia	Phosphotransferases
		e.g. ANT(2'')-Ia	Nucleotidyltransferases
	Target alteration	ArmA	16 ribosomal RNA methylase
Chloramphenicol		CAT-1	Acetyltransferase
Penicillins	β-lactam hydrolysis	e.g. TEM-1, CARB-5, SCO-1	Narrow-spectrum class A β-lactamases
		e.g. TEM-92, SHV-12, PER-1	Extended-spectrum class A β-lactamases.
Tetracycline	Active efflux	Tet(A), Tet(B)	Prevalent in European Clones I and II
Broad resistance (inc. quinolones and aminoglycoside)	Active efflux	AdeABC	Present in most *A. baumannii* strains

However it is the remarkable ability of *A. baumannii* to rapidly develop multi-drug resistance that appears to set it apart from not only other *Acinetobacter* species, but arguably also from other Gram-negative bacteria. So whilst the individual mechanisms of resistance to antibiotics are generally well known, the factors contributing to the success of *A. baumannii* in particular at rapidly developing multi-drug resistance and prevalence in the hospital environment are not clear.

Because of the frequent isolation of not only *A. baumannii* but other *Acinetobacter* spp from the clinical environment, it is important to investigate the ability of all of these strains to develop multi-drug resistance, to gain a true picture of the clinical situation.

1.4 Fluoroquinolone Resistance

The modes of action of the major antibiotic classes are shown in Table 1.1. Amongst these classes, fluoroquinolones have had the greatest clinical impact of the synthetic antibacterials, and are one of the largest classes of antimicrobial agents in worldwide use (Ruiz, 2003). However, due to rising bacterial resistance, perhaps the most dramatic example of which is resistance to fluoroquinolones (Livermore, 2007), there is an increasing call for a more cautious and educated use of these antimicrobials, alongside surveillance, in order to maintain their efficacy and that of those developed in the future (Applebaum & Hunter, 2000; Emmerson & Jones, 2003; Ruiz, 2003; Van Bembeke *et al*, 2005;).

1.4.1 Development of Fluoroquinolones

Quinolones, from which fluoroquinolones have evolved, originated as a modified compound isolated from the antimalarial agent chloroquine (Lesher *et al*, 1962*)*. Patented in 1962, nalidixic acid was the first quinolone, still in use against urinary tract infections (UTIs). Further iterations were developed with the addition of fluorination leading to use of the first fluoroquinolone, norfloxacin, in 1978. Subsequent iterations included ciprofloxacin in 1981 (Appelbaum & Hunter, 2000). Ciprofloxacin has a broad spectrum and can be used systemically, is one of the most potent fluoroquinolones against Gram-negative bacteria, and is still used for the treatment of a wide range of infections (Emmerson & Jones, 2003).The fluoroquinolone class of antibiotics has therefore evolved from the limited use of the original quinolone to a large class of broad spectrum, systemic antibiotics, with a huge worldwide market. However, it became apparent even in the first decade of use that there was a rapid emergence of bacterial resistance to ciprofloxacin (Thomson, 1999) and to quinolones in general (Applebaum & Hunter, 2000).

1.4.2. Mode of Action of Fluoroquinolones

Fluoroquinolones interact with two bacterial enzymes, DNA gyrase and DNA topoisomerase IV, inhibiting their action. DNA gyrase is a tetramer composed of two GyrA and two GyrB subunits, encoded by the *gyrA* and *gyrB* genes respectively. Topoisomerase IV is likewise comprised of two subunits each of ParC and ParE, encoded by the *parC* and *parE* genes (Drlica & Zhao, 1997).

Both enzymes are essential for bacterial DNA replication, and their inhibition therefore leads to the bactericidal effect of fluoroquinolones (Drlica & Zhao, 1997). DNA gyrase binds to DNA as a tetramer and introduces negative superhelical twists into DNA (supercoiling), necessary for initiating DNA replication (Roca *et al*, 1995). It also removes positive superhelical twists ahead of the replication fork, thereby facilitating the movement of replication and transcription complexes through DNA, allowing DNA replication. Topoisomerase IV acts in the later stages of DNA replication and separates the interlinked daughter chromosomes by decatenation, enabling segregation into daughter cells (Zechiedrich *et al*, 1995).

Fluoroquinolones inhibit these targets by binding to and stabilizing the cleaved complexes formed between the enzymes and their substrate DNA. The trapped DNA-enzyme complex blocks the activities of the enzymes, hence preventing DNA replication. This inhibition by fluoroquinolones is not thought to be lethal in itself; the bactericidal effect itself is thought to arise from the gyrase-mediated release of DNA ends, creating the equivalent of lethal double-stranded DNA breaks (Zhao *et al*, 1997). Different fluoroquinolones have varying affinities for the two target enzymes, and this is also dependant on the bacterial target in question. The older fluoroquinolones tend to have a higher potency against DNA gyrase than topoisomerase IV in Gram-negative bacteria, and vice versa in Gram-positive bacteria. The newer fluoroquinolones tend to have a more even activity against both targets (Hooper, 2000).

1.4.3 Bacterial Resistance to Fluoroquinolones

Bacterial resistance to fluoroquinolones is mediated by two main mechanisms: reduced drug concentration in the bacterial cell, associated with decreased permeability and over-expression of efflux mechanisms, and mutations leading to alteration in fluoroquinolone targets, preventing the action of these antibiotics. Whereas a decreased drug concentration allows for immediate survival and may be inducible the target site mutations are stable, and are therefore the primary mechanism of fluoroquinolone resistance (Van Bembeke, 2005).

Target site mutations most commonly occur in the *gyrA* and *parC* genes, and as the affinity of fluoroquinolones for the two targets varies between different bacteria, so the specific mutations associated with resistance vary. The commonly reported mutations, however, fall within the quinolone resistance determining regions (QRDRs) of these genes, comprising amino acids 67 to 106 (numbering for *Escherichia coli*) as first reported in *E. coli* (Yoshida, 1990).

1.4.4 Clinical Importance of Bacterial Resistance to Fluoroquinolones

Not only does fluoroquinolone resistance development impact upon the ability to treat patients and upon the future clinical usefulness of this class, it may also be linked with development of multi-drug resistance through associated over-expression of efflux, as

discussed for *Acinetobacter* (Section 1.4.6; Poole, 2001; Higgins *et al*, 2004; Van Bembeke *et al*, 2005; Vila *et al*, 2007). Target site mutations in *gyrA* and *parC* have also been found to be more frequent in outbreak-related than sporadic *Acinetobacter* strains (Wisplinghoff, 2003). There is also evidence that outbreak associated *A. baumannii* strains are significantly more resistant to fluoroquinolones than those associated with sporadic infections (Heinemann *et al*, 2000) agreeing with the suggestion that resistance to fluoroquinolones is a risk factor for epidemic behaviour in *A. baumannii* (Koeleman, 2001). Additionally, outbreak strains of *A. baumannii* with high fluoroquinolone MICs developed target site mutations in parallel with up-regulation of the *AdeB* efflux pump, which is also associated with resistance to other antibiotics (Higgins *et al*, 2004; Magnet *et al*, 2001).

1.4.5 Fluoroquinolone Resistance in *Acinetobacter* spp

In *Acinetobacter*, fluoroquinolone resistance is likewise associated with mutations within the QRDR of the *gyrA* and *parC* genes. Since Vila's work in the 1990s, which looked at the target site mutations of *Acinetobacter* that developed in response to challenge by ciprofloxacin, *gyrA* has been considered the primary target in *Acinetobacter*, with the Ser83 to Leu mutation being the most common. *parC* was considered the secondary target, with the Ser80 to Leu mutation being the most common (Vila, *et al* 1995; Vila *et al*, 1997). As such, resistance to fluoroquinolones was considered to develop in a stepwise fashion with a mutation in *gyrA* associated with low

level fluoroquinolone resistance and high level resistance being achieved when a *parC* mutation was also present.

In *gyrA* the loss of the *HinfI* restriction site GANTC at codons 82 and 83 when the most common position for target site mutation is affected (serine-83) allows for analysis with *HinfI* restriction enzyme; the possible existence of a target site mutation is indicated by unrestricted *gyrA* PCR products as seen on an agarose gel. However, since there are many substitutions at this site which could give an unrestricted product yet not all are associated with fluoroquinolone resistance (Waters & Davies, 1997), sequencing is advocated to confirm the indications of restriction analysis. This method, however, provides a useful tool for the quick assessment of clinically significant ciprofloxacin resistance in a selection of isolates.

Despite the importance of *gyrA* target site mutations as a stable bacterial resistance mechanism to fluoroquinolones, it has become apparent that the situation for *parC* and the development of high-level resistance is not so clear-cut: clinical *A. baumannii* isolates with high moxifloxacin MICs have been reported with a *gyrA* target site mutation but no *parC* target site mutation (Spence & Towner, 2003); novel mutations in both *gyrA* and *parC* have been reported in ciprofloxacin resistant *A. baumannii* isolates (Hamouda & Amyes, 2004); and high ciprofloxacin resistant laboratory-generated mutants have been reported with no *parC* mutations (Hamouda & Amyes, 2006).

Additionally, quinolone MICs can vary for strains with the same target site mutations (Wisplinghoff *et al*, 2003). These and other results have brought into question the importance of *parC* as a secondary target for fluoroquinolones. It is also considered that other mutations and mechanisms such as porin expression, outer membrane impermeability and efflux pumps contribute to fluoroquinolone resistance. Of these, efflux is increasingly recognised as playing an important role in the development of fluoroquinolone resistance in Gram-negative bacteria. Indeed, efflux may be a previously overlooked factor in earlier reports of high level fluoroquinolone resistance (Poole, 2004).

1.4.6 Efflux-mediated Resistance

In Gram-negative bacteria efflux systems often have broad substrate specificity, thought to work synergistically with the low permeability of the outer membrane to reduce antibiotic accumulation and leading to the development of multi-drug resistance (Poole, 2002). In *Acinetobacter* the AdeB multidrug efflux pump has been described (Magnet *et al*, 2001), with substrates including aminoglycosides, cefotaxime, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones, and ethidium bromide (Vila, 2007). The parallel appearance of target site mutations and up-regulation of the *adeB* gene was observed in outbreaks of *A. baumannii*, highlighting this organism's ability to rapidly develop multi-drug resistance (Higgins et al, 2004).

Whilst the AdeB pump confers resistance to most of these compounds, it was found that a further mechanism (presumed to be target site mutations) was necessary for fluoroquinolone resistance (Magnet et al, 2001). This supports the concept of fluoroquinolone target site mutations as the primary resistance mechanism; AdeB is associated with reduced susceptibility, rather than resistance, to fluoroquinolones.

It is unclear whether efflux pump over-expression is directly induced by antibiotics (Poole, 2001), but it seems likely that efflux may contribute to the emergence of resistant mutants by enabling survival even in sub-optimal concentrations of antibiotics (Van Bembeke *et al*, 2003), as has been found in *Pseudomonas aeruginosa* (Lomovskaya *et al*, 1999), potentially allowing the development of the stable target site mutations. The role of efflux in fluoroquinolone resistance many help to explain previously mentioned results (Spence & Towner, 2003; Wisplinghoff *et al*, 2003; Hamouda & Amyes, 2004; Hamouda & Amyes, 2006) where there is variation in MICs of isolates with the same target site mutation.

Whilst the development of multi-drug resistance mediated by efflux is of grave concern, and despite efflux being increasingly considered an important contributing factor in terms of fluoroquinolone resistance in *Acinetobacter*, it is the target site mutations that are the primary mechanism for resistance development, being a stable mutation rather than a reversible over-expression.

1.5 The Role of Hypermutation in the Development of Antibiotic Resistance

As discussed in Section 1.1, the rise of antibiotic resistance in clinical bacterial populations represents a problem of increasing importance with worldwide implications. The development of antibiotic resistance observed with the increased use of antibiotics is a paradigm of the Darwinian principles of survival of the fittest and the importance of variation; with their short generation time bacteria are able to rapidly adapt to new environments and stressful conditions, such as challenge with antibiotics. In particular, the remarkable ability of *Acinetobacter* to rapidly develop multi-drug resistance is crucial to its predominance as an opportunistic nosocomial pathogen. Evolution through genetic variation and selection is fundamental to this ability. It introduces new genes and alleles into the population and allows continual resistance development.

1.5.1 Mechanisms of Resistance Development

As mentioned in Section 1.1.2, bacterial resistance arises through the development of variation via horizontal transfer or mutation. Horizontal transfer and mutation may also act synergistically; mutation may produce new variations of alleles that have been acquired by horizontal transfer. Some examples of mutation in bacterial resistance development are detailed in Section 1.5.2.

1.5.2 The Importance of Mutation in Resistance Development

There are several antibiotic resistance mechanisms which involve mutation, demonstrated with the following examples. As discussed in Section 1.4.3, resistance to fluoroquinolones is mediated primarily via mutations in the *gyrA* and *parC* genes, which prevent the action of these agents. Mutation can also lead to overproduction of bacterial antibiotic-inactivating enzymes such as the cephalosporinase AmpC. Chromosomally encoded and usually produced at low-levels, antibiotic treatment can lead to the selection of de-repressed mutant cells producing high levels of AmpC, as observed in *Enterobacter* spp. isolates following use of broad-spectrum cephalosporins (Kaye *et al*, 2001).

A further effect of mutation upon the expression of 'intrinsic' resistance mechanisms is exemplified in efflux systems; over-expression of efflux can enhance resistance to fluoroquinolones and other agents and may be caused by mutations in regulatory genes, as in the efflux systems of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Poole, 2001; Schneiders *et al*, 2003). As mentioned (Section 1.4.6), increased expression of efflux systems can be associated with multi-drug resistance and as such the mutations that lead to the over-expression of efflux systems are similarly implicated. This is typified in *Pseudomona aeruginosa*, where raised MICs of antibiotics including fluoroquinolones, β-lactams and tetracyclines were associated with up-regulation of the mexA-mexB-oprM operon by mutation of *mexR*. As such multidrug resistance can be promoted as a result of mutational over-expression of the efflux genes (Poole, 2001).

It is thought that horizontal transfer and mutation work together in many cases of antibiotic resistance development (Blázquez, 2003), for example mutation is important for the evolution of acquired resistance genes; whilst their dissemination is mediated by acquisition via horizontal transfer, mutation of these genes is essential for diversification and hence their continued ability to provide bacterial resistance to antimicrobial agents. This is exemplified by the ever-expanding group of class-D OXA carbapenemases of *Acinetobacter*; these enzymes are associated with the emergence of carbapenem resistance in *A. baumannii* and their diversification via mutation is fundamental to their prevalence worldwide (Brown & Amyes, 2006; Evans *et al*, 2008).

Finally, mutation can be the predominant resistance development mechanism for a species, for example in *Mycobacterium tuberculosis* where resistance to all therapeutic agents is mediated by mutation rather than horizontal transfer (Gillespie, 2002).

Whilst the emergence of antibiotic resistance is a complex process often involving both horizontal transfer and mutational events, and varies depending on different antimicrobial agents and bacteria, it is apparent that mutation is an important aspect of the development and evolution of resistance.

1.5.3 The Importance of Mutator Cells in Resistance Development

The importance of mutation in resistance development has led to the theory that an increase in the bacterial mutation rate would increase bacterial resistance development by increasing the chance of favorable mutations developing. Early work with *Escherichia coli* led to increased interest in the potential importance of mutator cells in bacterial populations: Mutator genes were found to confer a selective advantage on a population containing them (Gibson *et al,* 1970); the appearance of new mutants in a population was suggested to be an essential part of the evolution of bacteria, with spontaneous mutations arising from errors in DNA replication, recombination or repair (Cox *et al,* 1972); and it was demonstrated that an advantageous mutation may become fixed in a bacterial population, linking the associated mutator with it, now termed 'hitch-hiking' (Cox, 1976).

Mutator cells and hypermutation have been identified in natural populations of several different species of bacteria. Reports include a high incidence of mutators in *Escherichia coli* and *Salmonella enterica* outbreak isolates (LeClerc *et al,* 1996), a high frequency of mutators in human uropathogenic *E. coli* isolates (Denamur *et al,* 2002) and mutators in both pathogenic and commensal strains of *E. coli* (Matic *et al*, 1997). A high frequency of mutators has also been reported in Serogroup A *Neisseria meningitides* isolates (Richardson *et al,* 2002) and *Haemophilus influenzae* and *Pseudomonas aeruginosa* isolated from the CF lung (Watson Jr. *et al,* 2004; Oliver *et al,* 2000).

Hypermutators have been most commonly associated with defects in the mismatch repair (MMR) system (detailed in Section 1.5.4), determined through analysis of clinical isolates (LeClerc *et al*, 1996; Matic *et al*, 1997; Watson Jr. *et al*, 2004; Oliver *et al*, 2000) and also demonstrated in laboratory studies where defective MMR has been associated with hypermutation and/or resistance development in bacteria including *E. coli, Salmonnellae* spp, *Staphylococcus aureus, P. aeruginosa* and *A. baumannii* (Zahrt *et al*, 1994; Mao *et al*, 1997; Oliver *et al*, 2002; Miller *et al*, 2002; Schaaff *et al*, 2002; O'Neill & Chopra, 2002; Young & Ornston, 2001).

Whilst it is apparent that mutators exist in the natural populations of several species of bacteria, their role in antibiotic resistance development is subject to some debate. There has been increasing interest and a great deal of research into the existence and relevance of mutators within bacterial populations in resistance development, including laboratory studies and animal modeling of mutator populations and dynamics. Some aspects of this area are summarized below.

Laboratory studies of *E. coli* have indicated an advantage conferred by increased mutation rates, with mutator cells increasing in populations compared to non-mutators, and proliferating in a population following selections with antibiotics, highlighting the potential for antibiotics to indirectly select for mutator cells (Chao, 1983; Mao, 1997). Computer simulation has indicated that mutator genotypes can be important in adaptive evolution, and may become fixed in the population by 'hitch-hiking' with the advantageous mutations they generate (Taddei *et al*, 1997). This has also been shown

using a mouse model, demonstrating the selection of mutator bacteria present at low frequencies in wild type *E. coli* populations during a course of antibiotic treatment, the mutator allele being selected as the mechanism that generated the antibiotic resistance (Giraud *et al*, 2002).

There is some debate about whether mutator cells are always present in populations or are the result of a transient increase in mutation rate (Chopra *et al*, 2003). Whilst it had been thought that the mutation rate of bacterial populations would evolve to be as low as possible due to the accumulation of deleterious mutations and fitness costs associated with the mutator phenotype (Funchain *et al*, 2000), the frequent occurrence of mutator cells isolated from naturally occurring bacterial populations questions this assumption. It is thought that the existence of mutators in a population may be due to their recent passage through a bottleneck, leading to cells within the population with increased mutation rates and associated beneficial mutations being selected (Chopra *et al*, 2003). Deficiencies in the MMR system, described in more detail in Section 1.5.4, are frequently associated with mutator phenotypes and this is thought to be an important aspect in the balance between variation and stability (Woodford & Ellington, 2007).

There is evidence that the initial fitness costs associated with mutators may be limited by compensatory mutations arising. This has been demonstrated in the mouse model study described above, where it was found that compensatory mutations were rapidly accumulated (Giraud *et al*, 2002). Also, compensatory mutations were found to be common in *gyrA* and *parC* mediated quinolone resistant *Pseudomonas aeruginosa*

isolates, increasing their fitness (Kugelberg *et al*, 2005). Additionally, fitness costs may be attenuated by adaptation during multiple passages (Björkman *et al*, 2000). Hence it seems possible that mutator cells can exist in populations and that the elevated mutator rates of hypermutators in a bacterial population could increase the frequency of antibiotic resistance development, whilst also increasing the chance of parallel compensatory mutations arising to reduce fitness costs associated with the increased mutation rate.

However, higher mutation rates also do not necessarily result in an increased ability to develop resistance (Matic *et al*, 1997; Denamur *et al*, 2002; O'Neill & Chopra, 2002;) and there is concern about the different uses of mutation rates and the variance in data from different studies (Martinez & Baquero, 2000; Woodford & Ellington, 2007). Therefore there must be caution in considering a higher mutation rate in isolation as a cause of resistance development.

Whilst there is a potential role for hypermutation in resistance development, the relevance of hypermutators in the clinical environment can be contentious. However hypermutation is considered a key factor in the development of resistance in *P. aeruginosa* isolates in the CF lung and other chronic infections. There are reports of high frequencies of hypermutable *P. aerunginosa* strains in patients with cystic fibrosis and other chronic lung infections, compared with populations of acute infections. Mutators were most commonly MMR-deficient with the *mutS* gene implicated, were associated with substantially higher resistance rates, and were associated with multi-drug

resistance. This has led to hypermutation being considered a key factor for multi-drug resistance development of *P. aeruginosa* and its long-term persistence (Oliver *et al*, 2000; Oliver *et al*, 2002; Oliver *et al*, 2004; Maciá *et al*, 2005). In addition, a recent study reported a higher than expected prevalence of mutators in early infection and environmental isolates, which are thought to be the primary source of infection (Kenna *et al*, 2007).

Whether hypermutation is a result of pre-existing mutator cells or transient hypermutation, or more likely a complex interplay between these and other aspects of bacterial population dynamics, the documented existence of hypermutators in natural bacterial populations, their association with antibiotic resistance in both laboratory and clinical isolates, and their potential relevance in the clinical environment is undoubtedly an important area of research.

1.5.4 The Mismatch Repair System and Role in Hypermutation

Genetic change mediated by mutation in bacteria occurs predominantly through errors arising during DNA replication. Of the mechanisms involved in regulating this process, the most important is the mismatch repair (MMR) system, crucial to avoid mutations and maintain the integrity of the genome. Hence, defects in MMR lead to associated replication errors and increase the spontaneous mutation rate, and are the mechanism most frequently associated with hypermutation in bacterial populations (Hsieh, 2001; Li,

2008), as mentioned above in Section 1.5.3. MMR is a highly conserved pathway, especially well characterized in *E. coli*, and its function in mismatch correction is now known to require several proteins in addition to the principal proteins MutS, MutL and MutH (Li, 2008).

The primary function of MMR is to carry out post-replication repair, correcting mismatches that arise during DNA replication. It can also function as a barrier to interspecies recombination, by preventing the recombination process between similar but not identical sequences (Rayssiguier *et al*, 1989), hence its inactivation can lead to a potential increase in horizontal transmission between species (LeClerc *et al*, 1996). MutS, homologues of which have been found throughout prokaryotes and eurkaryotes, initiates MMR by recognising and binding specifically to base/base and insertion/deletion mismatches (Obmolova *et al*, 2000; Lamers *et al*, 2000; Lamers *et al*, 2003). Recent work using atomic force microscopy has further elucidated the mechanism of MutS searching for and recognising mismatches (Tessmer *et al*, 2008). In the presence of ATP, MutL then interacts with MutS and activates MutH. MutH then specifically cleaves the transiently unmethylated daughter strand at hemimethylated GATC sequences (Hsieh, 2001).

The importance of MMR, and in particular MutS, is apparent from the prevalence of mutator phenotypes associated with MMR-deficiency via defects in the *mutS* gene; defective *mutS* has been associated with mutators in clinical isolates of bacteria including *E. coli, S. enterica* and *P. aeruginosa* (LeClerc *et al*, 1996; Watson Jr. *et al*,

2004; Oliver *et al*, 2002). An elevated mutation frequency due to *mutS*-knockout has been shown to correlate with the development of vancomycin resistance in *S. aureus* isolates (Schaaff *et al*, 2002), and mutations in the *mutS* gene of *Enterococcus faecium* isolates have also been correlated to linezolid resistance, even in the absence of detectable hypermutation (Willems *et al*, 2003). Additionally inactivation of MMR has been demonstrated to overcome the barrier in transduction between *Salmonella typhimurium* and *Salmonella typhi* (Zahrt *et al*, 1994), due to its function as a barrier to interspecies recombination. Phylogenetic studies have also indicated that there is horizontal transfer of the *mutS* gene of naturally occurring *E. coli*, consistent with a possibility of transient hypermutation via the balance of adaptive changes by mutation and subsequent rescue of defective *mutS* alleles (Denamur *et al*, 2000; Brown *et al*, 2001).

1.5.5 The *mutS* gene of *Acinetobacter*

In *Acinetobacter* there has to date been no study of MMR-deficiency in clinical isolates. However, Young and Ornston have characterized the *mutS* gene from *Acinetobacter* sp. strain ADP1 (Young & Ornston, 2001). ADP1, sometimes referred to as BD413, is a derivative of the soil isolate *Acinetobacter* sp. strain BD4, and has since been classified as a member of the recently established species *Acinetobacter baylyi*. ADP1 and other members of this species are characterized by a natural transformation system, resulting in an unusual potential for acquiring foreign DNA (Vaneechoutte *et al*, 2006).

Comparison of the *mutS* sequence of ADP1 with other Gram-negative bacteria identified a substantial sequence correlation, especially within the mismatch-binding and helix-turn-helix domains, but identified six indels present in the *mutS* sequence of ADP1. Several other *Acinetobacter* strains examined also had these indels, separating *Acinetobacter* from other Gram-negative bacteria, with indel one also separating different *Acinetobacter* strains. Inactivation of the *mutS* gene was shown to increase the frequency of rifampin resistance mutations in ADP1 to 54-fold greater than that of the wild-type strains. Additionally, inactivation of *mutS* significantly increased the transformation frequencies for most divergent donors, as has been found in other organisms (Young & Ornston, 2001).

These results suggest that hypermutation, and particularly defective *mutS* causing malfunctioning MMR, may be a factor in the ability of clinical *Acinetobacter* to rapidly develop antibiotic resistance, as has been observed in other bacteria.

1.6 Summary, Hypotheses and Aims

- Both Gram-positive and Gram-negative nosocomial bacteria are current causes for concern in the clinical environment, as exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter* spp, particularly *A. baumannii*.
- The rise of multi-drug antibiotic resistance and the associated threat to treatment and control options is the principal factor leading to increased concern.

1.6.1 *Acinetobacter* Summary

- The *Acinetobacter* genus is diverse and has a complex taxonomic history, traditionally making speciation difficult.
- *A. baumannii* is the species of primary clinical importance, although other species are also isolated in the clinical environment and there is increasing recognition of the importance of other species, especially genospecies 13TU.
- The ability to rapidly acquire multi-drug resistance is fundamental to the success of *A. baumannii* in particular as an opportunistic pathogen; whilst individual resistance mechanisms are known, factors contributing to this ability are unclear.
- Hypermutation and mutator cells are increasingly considered an important factor in the development of antibiotic resistance in several bacterial species, particularly *Pseudomonas aeruginosa*.
- Defects in the *mutS* gene have been associated with hypermutation via deficient mismatch repair, increased ability to develop resistance, and increased

transformation frequencies, but there has been no investigation of clinical isolates to date.

- Inactivation of the *mutS* gene of the non-clinical *Acinetobacter baylyi* strain ADP1 led to increased frequency of rifampin resistance mutations and increased transformation frequencies.
- Increased mutation frequencies alone do not necessarily predict increased antibiotic resistance development.
- Fluoroquinolone resistance is a risk factor for epidemic behaviour in *Acinetobacter* and is primarily mediated by target site mutations in the *gyrA* gene.

1.6.2 Hypothesis:

That genetically distinct sub-populations of *Acinetobacter* spp clinical isolates exist, with variations in the *mutS* gene, able to more rapidly develop antibiotic resistance through defective mismatch repair.

1.6.3 Aims

- Speciation of clinical *Acinetobacter* spp isolates.
- Characterisation of the *mutS* gene of clinical *Acinetobacter* spp isolates with varying antibiotic susceptibilities.
- Comparison of the *mutS* gene of clinical *Acinetobacter* spp isolates between each other and against non-clinical strains: is there *mutS* gene variation?

- Analysis of the *mutS* gene of clinical *Acinetobacter* spp isolates in terms of sensitivity: does any variation in the *mutS* gene of clinical isolates correlate with varying antibiotic susceptibility?
- Mutation studies to determine the ability of sensitive and intermediate-resistant *Acinetobacter* spp clinical isolates to develop antibiotic resistance, measured by development of resistance to ciprofloxacin and comparison to resistant isolates: is any variation in the *mutS* gene of clinical isolates associated with varying ability to develop resistance?

1.6.4 MRSA Summary

- Increasing antibiotic resistance in MRSA and the emerging threat of CA-MRSA in the hospital environment means infection control is an essential tool.
- With resurgence of infection control has come an increase in the use of biocides such as chlorhexidine, both in the clinical and domiciliary environment.
- Whilst in-use biocide concentrations are high, they may be present at low concentrations in the hospital environment.
- Low concentrations of biocide have been found to select for reduced susceptibility isolates and associated antibiotic resistance.

1.6.5 Hypothesis:

That low concentrations of chlorhexidine exert selective pressure on clinical MRSA isolates and are less effective against clinical isolates than standard strains, leading to the development of reduced susceptibility to chlorhexidine associated with increased antibiotic resistance.

1.6.6 Aims

- To assess the efficacy of low concentrations of chlorhexidine upon current clinical MRSA isolates: is there variation in the susceptibility of clinical isolates?
- To compare the efficacy of low concentrations of chlorhexidine upon clinical MRSA isolates and standard strains: is chlorhexidine more effective against standard strains than against clinical MRSA isolates?
- To determine whether exposure of clinical MRSA isolates to chlorhexidine residues is associated with the development of antibiotic resistance.

Chapter 2: Materials and Methods

2.1 Bacterial Strains

Clinical *Acinetobacter* spp. isolates were collected from the blood culture collection of the Royal Infirmary Edinburgh (RIE) clinical labs, comprising isolates from 1995-1999. They were sub-cultured and stored at -70°C in Nutrient Broth (Oxoid, UK) containing 10% glycerol. Previously collected isolates from worldwide sources from the isolate collections of Dr Paul Higgins and Dr Susan Brown were also used. Standard strains and prevalent outbreak *Acinetobacter* strains were also included for comparison. These are detailed in Table 2.1 and some were kindly gifted by Dr Kevin Towner and Dr Jane Turton, as stated.

Table 2.1 – Type strains and other strains used

Strain	Number	Source
Acinetobacter calcoaceticus (sp. 1)	ATCC 23055	Kevin Towner
Acinetobacter baumannii (sp. 2)	ATCC 19606	Laboratory collection
Acinetobacter gen.sp. 3	ATCC 19004	Kevin Towner
Acinetobacter haemolyticus (sp. 4)	ATCC 17906	Kevin Towner
Acinetobacter junii (sp. 5)	ATCC 17908	Kevin Towner
Acinetobacter gen.sp. 6	ATCC 17979	Kevin Towner
Acinetobacter johnsonii (sp. 7)	ATCC 17909	Kevin Towner
Acinetobacter Iwoffii (sp. 8)	ATCC 5866	Kevin Towner
Acinetobacter radioresistens (sp. 12)	-	Kevin Towner
Acinetobacter gen.sp. 13TU	-	Kevin Towner
A. baumannii NW strain	JTA	Jane Turton
A. baumannii W strain/European clone 1	JTB	Jane Turton
A. baumannii Midlands 2	JTC	Jane Turton
A. baumannii T strain	JT3	Jane Turton
A. baumannii SE Clone	JT4	Jane Turton
A. baumannii OXA-23 Clone 2	JT6	Jane Turton
Escherichia coli	NCTC 10418	Laboratory collection
Pseudomonas aeruginosa	NCTC 10662	Laboratory collection
Staphylococcus aureus	NCTC 6571	Laboratory collection

The *A. baumannii* type strain ATCC 19606 and the environmental *A. baylyi* strain ADP1 (also known as BD413) were also included as controls during the study. The *mutS* sequences of further non-clinical control strains (Young & Ornston, 2001) were also used during sequence analysis. Strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* were used as controls in MIC determination, as detailed in Section 2.7.

Clinical MRSA samples were collected from the New Royal Infirmary Edinburgh (NRIE) clinical labs from February to April 2006, sub-cultured on blood agar and stored at -70°C on beads. Only hospital-acquired MRSA isolates (isolated after 48 hours of admission) were included. The prevalent Epidemic MRSA (EMRSA) strains 15 and 16 were gifted by Dr Leila Vali for use in this study.

2.2 Broths and Agars

Unless otherwise indicated, all broths and agars were obtained from Oxoid (Basingstoke, UK), reconstituted with distilled water in the lab according to the manufacturer's instructions and autoclaved before use to ensure sterility. The broths and agars used are detailed in Table 2.2

Table 2.2 Abbreviations of commonly used broths and agars

Component	Abbreviation
Nutrient Agar	NA
Nutrient Broth	NB
MacConkey Agar	MC
Iso-sensitest Agar	IST agar
Iso-sensitest Broth	IST broth

2.3 Reagents and Buffers

Unless otherwise stated, reagents and components of buffers were obtained from Sigma-Aldrich, Poole, UK. Commonly used solutions are detailed in Table 2.3 below. Primers used in PCR reactions are detailed in Table 2.4.

Table 2.3 Solutions and their components

Solution	Components
Saline (0.85%)	4.5 g NaCl in 500 ml SDW, autoclaved.
TAE buffer	40 mM Trizma base, 20 mM EDTA (disodium), in SDW, pH 8
10 x TBE buffer	1.0 M Trizma base, 0.9 M Boric acid, 10mM EDTA (disodium), in SDW, pH 8.2-8.4, autoclaved.
TE buffer	10mM Tris-HCl, 5mM EDTA, in SDW, pH 7.5, autoclaved.
ES buffer	0.5mM EDTA, 10% Sarkosyl, in SDW, pH 8, filter sterilised, 1mg/ml Proteinase K added before use.
Agarose LMP	BioGene
Agarose	BioGene
Neutraliser	0.75% (w/v) azolectin and 5% Tween 80 in SDW, autoclaved

Table 2.4 Primers used for *mutS* PCR

PCR	Section	Primers	Nucleotide Sequence (5'-3')	Reference
tDNA	2.6.1.1	T3A	GGGGGTTCGAATTCCCGCCGGCCCCA	Welsh & McClelland, 1991
		T5B	AATGCTCTACCAACTGAACT	
OXA-51-like	2.6.1.2	OXA-51-likeF	TAATGCTTTGATCGGCCTTG	Turton *et al*, 2006
		OXA-51-like-R	TGGATTGCACTTCATCTTGG	
mecA	2.6.2	mecA1	GTAGAAATGACTGAACGTCCGATA	Vali *et al*, 2008
		mecA2	CCAATTCCACATTGTTTCGGTCTA	
mutS	2.9.1	LH1/C	TAYMGIATGGGIGAYTTYTAYGA	Designed by Dr Susan Brown, this study
		RH1/F	TTIKGRTCIACIADRTYYTCRTC	
gyrA	2.10.3	gyrA1	AAATCTGCCCGTGTCGTTGGT	Vila *et al*, 1995
		gyrA2	GCCATACCTACGGCGATACC	

2.4 Antimicrobial agents

Antibiotics and other antimicrobial agents used are detailed in Table 2.5

Table 2.5 Antimicrobial agents, their abbreviations and sources

Antimicrobial		Source
Ampicillin	AMP	Sigma-Aldrich, Poole, UK
Augmentin (co-amoxyclavulanic acid)	AUG	Sigma-Aldrich, Poole, UK
Cefotaxime	CTX	Roussel Laboratories, Romainville, France
Cefuroxime	CEF	Sigma-Aldrich, Poole, UK
Ceftazidime	CAZ	GlaxoSmithKline, Stevenage, UK
Chlorhexidine diacetate hydrate	CHX	Sigma-Aldrich, Poole, UK
Chlorhexidine digluconate	CHG	Sigma-Aldrich, Poole, UK
Ciprofloxacin	CIP	Bayer AG, Leverkusen, Germany
Gentamicin	GEN	Sigma-Aldrich, Poole, UK
Imipenem	IMP	MerckSharpeDohme, NJ, USA
Meropenem	MER	Zeneca Pharm., Macclesfield, UK
Oxacillin	OXA	Sigma-Aldrich, Poole, UK
Tetracycline	TET	Sigma-Aldrich, Poole, UK
Vancomycin	VAN	Sigma-Aldrich, Poole, UK

2.5.1 Preparation of DNA for PCR – *Acinetobacter* spp

After growth of a sample of the stored isolate on an MC agar plate overnight, a single colony was resuspended in 50 µl sterile MilliQ water and boiled for 10 minutes. Following snap-cooling on ice and a brief centrifugal step, the supernatant was then used in subsequent PCR reactions.

2.5.2 Preparation of DNA for PCR - MRSA

DNA was prepared using the rapid lysis procedure (Ünal, 1992) whereby a 1µl loop of overnight growth was resuspended in 50µl 100µg/ml lysostaphin, incubated for 10 minutes at 37°C, 50µl of 100µg/ml Proteinase K and 150µl 0.1M Tris buffer were added and the mixture incubated for 10 minutes at 37°C followed by boiling for 5 minutes.

2.6.1 Speciation of *Acinetobacter* isolates

2.6.1.1 tDNA fingerprinting method

Speciation to genomic group level was performed by PCR amplification and restriction of tRNA spacer regions using a modified method (Ehrenstein *et al,* 1996). ATCC type strains (detailed in Table 2.1 above) were included to enable comparison. One pmole/µl aliquots of each primer T3A and T5B (Table 2.4) were added to a PCR mixture comprising 2µl DNA (from lysate) and PCR components from Promega: 1 x buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 2 units of Taq DNA polymerase, with SDW to give a final volume of 50µl.

PCR mixtures were cycled in a Hybaid Px2 Thermal Cycler under the following conditions: 94°C for 2 minutes; 45 cycles of: 94°C for 40 seconds, 50°C for 40 seconds, 72°C for 2 minutes; 72°C for 3 minutes. Products were run on a 2% agarose gel (biogene) in 1xTAE buffer at 120V for 90 minutes, stained with ethidium bromide, and visualised under UV light (Bio-Rad Gel Doc 2000, Bio-Rad). Band patterns were

compared to reference strains using the BioNumerics™ software package (version 3.0, Applied Maths MVBA, Saint-Martens-Latem, Belgium).

2.6.1.2. OXA-51-like PCR

OXA-51-like genes were detected using a modified method (Turton *et* al, 2006). Template DNA was amplified using primers OXA-51-likeF and OXA-51-likeR (Table 2.4) in a 20µl PCR reaction containing HotStarTaq Plus Master Mix (Qiagen). PCR mixtures were cycled in a Hybaid Px2 Thermal Cycler under the following conditions: 95˚C for 5 minutes; 30 cycles of: 94˚C for 25 seconds, 52˚C for 40 seconds, 72˚C for 50 seconds; 72˚C for 6 minutes. Products were run on a 1.5% agarose gel (biogene) in 1xTAE buffer at 100V for 40 minutes, stained in ethidium bromide, and visualised under UV light (Bio-Rad Gel Doc 2000, Bio-Rad). The presence of OXA-51-like was indicated by a band of 353bp.

2.6.2 Confirmation of Presence of *mecA* in MRSA Isolates

Isolates collected from the NRIE clinical labs were confirmed as MRSA by PCR of the *mecA* gene. Ten µl lysate was used in a *mecA* PCR reaction of final volume 50µl comprising SDW, 25µl HotStar Taq (Qiagen) and 10pmole each primer MecA1 and MecA2 (Table 2.4). PCR mixtures were amplified in a Hybaid Px2 Thermal Cycler (Thermal Hybaid, Ashford, UK) according to the following cycling: 1 cycle of 15 minutes at 95°C; 30 cycles of: 1 minute at 94°C, 1 minute at 50°C, 1 minute at 72°C; 1 cycle of 10 minutes at 72°C. PCR products were run on a 1.5% agarose gel (Biogene)

against a *mecA* positive control by electrophoresis in TAE buffer at 100 volts, stained in 0.5 mg/ml ethidium bromide and visualized under UV light (Bio-Rad Gel Doc 2000, Bio-Rad).

2.7 Minimum Inhibitory Concentration (MIC) determination

MIC testing for a range of antibiotics was performed by the agar dilution method, according to the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for susceptibility testing (Andrews, 2001; MacGowan & Wise, 2001). To summarise, an overnight culture of each bacterial sample in IST broth (10^7-10^8 cfu/ml) was diluted in sterile 0.85% saline to approximately 10^4 cfu/ml and inoculated onto IST agar plates containing a range of antibiotic concentrations (0.002-256 mg/L), using a Denley Multipoint Inoculator to give 10^4 cfu/spot. Following overnight incubation the results were interpreted according to the BSAC guidelines (Andrews, 2001; MacGowan & Wise, 2001). *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* NCTC 10662, *Staphylococcus aureus* NCTC 6571 and *Acinetobacter baumannii* ATCC 19606 were used as control strains.

2.8 PFGE typing of *Acinetobacter* isolates

The protocol was that advocated by the Antibiotic Resistance Prevention And Control (ARPAC) project (Bannerman *et al*, 1995).

2.8.1 Plug Production

Isolates were subcultured onto MC agar plates and a suspension made into 2 – 3 ml TE buffer. Turbidity was adjusted to an optical density of 1.8 – 2.0 using a spectrophotometer at a wavelength of 540nm. 2% w/v low-melting point agarose in TE buffer was prepared and kept warm until use. Bacterial solution (500µl) was transferred into and Eppendorf tube, and 500µl melted agarose added and mixed. Aliquots of 150µl were dispensed into each plug mould and plugs allowed to set for 20 minutes at 4°C. Plugs were transferred into a Universal bottle containing 3 ml of ES solution with 1 mg/ml Proteinase K, then incubated at 55°C for 3 hours. Plugs were washed four times with 5 ml TE buffer for 3 hours, with one wash overnight, and stored at 4°C.

2.8.2 Restriction Digestion

A slice of each plug was cut and washed with TE buffer for 15 minutes. The TE was removed and plugs washed twice with *Apa*I buffer (Promega) for 20 minutes each time. *Apa*I buffer (200µl) and 20 units *Apa*I restriction enzyme was then added and plugs incubated at 30°C overnight.

2.8.3 Gel Preparation

A PFGE gel was prepared using 1.5% w/v agarose in 0.5xTBE. 0.5xTBE was added to the CHEF-DR II electrophoresis cell and chilled to 14°C before the beginning of the run. The restriction enzyme mixture was aspirated from the plugs and rinsed with 200µl 0.5 x TBE and the slices added to the wells in the gel, with strain RUH 2034 and a

lambda ladder being run alongside the samples. The wells were sealed with molten agarose and allowed to set for 10 minutes then loaded into the PFGE chamber.

2.8.4 Electrophoresis and Visualisation

PFGE was performed on the CHEF-DR II electrophoresis cell with a ramped pulse, initial 5 seconds, final 13 seconds, 200V for 20 hours at 14°C. Gels were then stained with ethidium bromide for 45 minutes and de-stained with SDW for several hours before being visualised under UV light (Bio-Rad Gel Doc 2000, Bio-Rad) and analysed using the BioNumerics™ software package (version 3.0, Applied Maths MVBA, Saint-Martens-Latem, Belgium).

2.9 Sequencing of the *mutS* gene of *Acinetobacter* isolates

2.9.1 PCR of the *mutS* gene

Degenerate primers were designed, by Dr Susan Brown, from areas of amino acid conservation in the *mutS* sequences of *Acinetobacter* sp. strains ADP1, 93A2, AD321, AC423D and LUH540 (Young and Ornston, 2001), and synthesized by MWG-Biotech AG (Cork, Ireland). Inosine was inserted to reduce degeneracy at positions where any of the four bases were required. Sections of the N-terminal region were PCR amplified. The PCR required optimisation with different combinations of primer pairs. The primers used are shown in Table 2.4.

One µl of template DNA was amplified using components from Promega, Southampton, UK in a PCR mixture containing: 10 x Buffer (10 mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, PCR Nucleotide Mix (containing 10 mM each dNTP), 2 pmol/µl each primer LH1/C and RH1/F, 2 units of *Taq* DNA polymerase and MilliQ water to give a final volume of 50 µl.

PCR mixtures were amplified in a Hybaid Px2 Thermal Cycler (Thermal Hybaid, Ashford, UK) according to the following cycling: 35 cycles of 95°C for 1 minute, gradient temperature of 35-50°C for 1 minute, 72°C for 1 minute; 1 cycle of 72°C for 5 minutes. Gradient cycling was used to allow a range of annealing temperatures to be used simultaneously for each reaction, hence facilitating the optimization of the annealing temperature variable for each isolate.

2.9.2 Gel Electrophoresis

PCR products were run on a 2% low-melting-point agarose gel (preparative grade, Promega) by electrophoresis in TAE buffer at 100 volts, stained in 0.5 mg/ml ethidium bromide and visualized under UV light (Bio-Rad Gel Doc 2000, Bio-Rad). A 100 bp DNA ladder was run alongside for visual sizing of the bands. The specific band (489 bp) was excised from the gel and purified using a gel extraction kit (Qiagen, Hilden, Germany).

2.9.3 Sequencing and Analysis

Sequencing of the purified products was performed by DNAShef, The Royal Infirmary, Edinburgh. Sequences were analysed by alignment using hierarchical clustering against control strains (Corpet, 1988).

2.10 Mutation Studies of *Acinetobacter* isolates

2.10.1 Generation of Mutants

Mutants were selected by challenge with ciprofloxacin. Parent strains were sub-cultured onto MC agar and a colony re-suspended in IST broth for 24 hours at 37°C. First-step mutants were selected in triplicate on IST agar containing 2 x parent MIC of ciprofloxacin. 100µl of neat broth was spread onto each of three plates and a 1:100 dilution of broth was spread onto a further three plates.

Non-selective IST plates were similarly inoculated to enable a viable count to be determined for each parental strain. The viable count plates were incubated for 24 hours at 37°C and colony counting performed. The selective plates were inoculated for up to 48 hours at 37°C and colony counting performed, using either the neat or 1:100 plates depending on which gave the appropriate colony numbers per plate. The mutation frequency was calculated as the ratio of potential mutants to viable colonies for each isolate.

Up to 10 mutants were picked from each plate, sub-cultured on non-selective MC agar, and stored in nutrient broth with 10% glycerol at -70°C. Ciprofloxacin MICs were determined for all picked mutants. Those with raised MIC values compared to their parent were used to generate second-step mutants as described above, by challenge with 2 x first-step mutant MIC of ciprofloxacin.

2.10.2 Mutant Stability

The stability of certain mutants of interest was determined by a tenfold passage on non-selective MC agar with mutants being considered stable if the MICs remained unchanged after the passaging.

2.10.3 PCR Amplification of the *gyrA* QRDR of parents and mutants

The *gyrA* QRDRs of first-step mutants and, where appropriate, second-step mutants were amplified. Parental strains were also amplified for comparison. Despite optimization, it was not possible to amplify all *gyrA* QRDRs to a sufficient degree of specificity using standard materials. However the use of HotStarTaq DNA Polymerase (Qiagen), to prevent the formation of mis-primed products and primer-dimers at low temperatures, increased the specificity of the PCR and enabled successful amplification of more of the isolates.

The *gyrA* QRDRs of samples were amplified in reactions containing: 30 pmol/µl each primer gyrA1 and gyrA2 (Table 2.4), 1 mM $MgCl_2$, 10 µl template DNA, 25µl HotStarTaq Master Mix (2.5 units HotStarTaq DNA Polymerase, 1 x PCR buffer, 200 µM each dNTP) with MilliQ water to give a final volume of 50 µl.

Reactions were amplified in a Hybaid Px2 Thermal Cycler (Thermal Hybaid, Ashford, UK) according to the following cycling: 95°C for 15 minutes; 32 cycles of 94°C for 1 minute, 57.5°C for 1 minute, 72°C for 1 minute; 72°C for 5 minutes.

Products were visualised as described previously on 1.5% agarose (BioGene Ltd, Kimbolton, UK) stained with Ethidium Bromide under UV light. A band of 343 bp indicated successful amplification of the *gyrA* QRDR.

2.10.4 *Hinf*I RFLP analysis of *gyrA* PCR Products

Target site mutations at position 83 in GyrA are associated with loss of the *Hinf*I restriction site in *gyrA* PCR products of *Acinetobacter* (Vila *et al*, 1995). To give an indication of whether target site mutations were present in the isolates, 10 µl of PCR product was added to 2 µl restriction enzyme buffer (10 mM Tris-HCl, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol), 0.2 µl bovine serum albumin (0.5 mg/ml) and 5 units HinfI (Promega), with MilliQ water to a final volume of 20 µl. Reactions were incubated for 2 hours at 37°C and the products run on a 1.5 % agarose gel and visualised under UV light as described previously.

An intact restriction site (i.e. no target site mutation) was indicated by two bands of 291 bp and 52 bp for *gyrA*. Restricted and unrestricted ATCC 19606 samples were run concurrently as controls.

2.10.5 Sequencing of *gyrA* PCR Products

To confirm any indication of target site mutations from the RFLP detailed above and determine whether any other changes were present in the sequences *gyrA* PCR products were purified using a PCR Purification Kit (Qiagen), sequenced as described previously, and aligned using hierarchical clustering (Corpet, 1988) against parent strains and the control ciprofloxacin-susceptible strain HCP-77 (Vila *et al*, 1995).

2.11 Biocides and MRSA

2.11.1 Preparation of Bacterial Cells

Where a washed suspension of bacterial cells was required, isolates were grown overnight in 10 ml nutrient broth at 37°C. Broths were centrifuged at 1500g for 10 minutes, the supernatant discarded, and the pellets washed twice in 10 ml saline. Cells were resuspended in saline for use in the tests, giving approximately 10^8 cfu/mL.

2.11.2 Preparation of Solutions

Chlorhexidine diacetate hydrate salt was dissolved in SDW using magnetic stirrers and heat, and filter sterilised through a 0.22µm filter before use. A stock solution was made and diluted appropriately for the required test concentrations.

Chlorhexidine digluconate, purchased in liquid form, was diluted to the appropriate concentration in SDW and filter sterilised as above.

The neutraliser solution comprised 0.75% (w/v) azolectin and 5% (v/v) Tween 80 dissolved in SDW and autoclave sterilised before use. The use of a neutraliser in the following tests is essential to quench the activity of the biocide and allow accurate biocide contact times to be tested.

2.11.3 Drop Counting Method

Counts for controls and tests were quantified using the drop counting method. 0.1ml of each sample was serially diluted in SDW and three 10µl drops spotted onto the surface of an over-dried NA plate. After overnight incubation at 37°C, colonies were counted and cfu/ml calculated.

2.11.4 Calculation of Microbiocidal Effect

Specific controls were performed concurrently with each biocide experiment to enable the calculation of a unique microbiocidal effect (ME) for that test. The ME is the log reduction in cell count after contact with biocide:

ME = log cfu/mL control – log cfu/mL test

where the control shall be explained for each experiment, but will generally consist of the same experimental influences except biocide is replaced by SDW.

As the ME measures the reduction in cell counts, it is specific and accurate for each test, and hence allows comparison of the effects of chlorhexidine between different isolates.

2.11.5 General Controls

Controls testing the efficacy and toxicity of the neutraliser were performed for each new stock made against the chlorhexidine concentrations to be used.

Neturaliser toxicity was evaluated by the addition of 1mL of neutraliser to the prepared bacteria, followed by 5 minutes contact time. Cells were then re-suspended, serially diluted, and counted using the drop counting method. The number of survivors was compared with those for a control where SDW replaced the neutraliser, any difference in counts giving an indication of the toxicity, if any, of the neutraliser.

Neutraliser efficacy was evaluated to ensure the biocide was being quenched as required. Neutraliser was added to the chlorhexidine and 5 minutes contact time allowed before the addition of bacteria and a further 5 minutes of contact time. Drop counting was performed and the count compared to a sample without biocide quenching, the neutraliser being replaced by SDW.

Significant differences between controls were examined using the t-Test to establish P values.

2.11.6 Quantitative Suspension Test

As traditional MIC testing is not appropriate for biocides quantitative suspension tests were used to determine the bactericidal activity of chlorhexidine (Thomas *et al*, 2005).

One ml of the washed bacterial inoculum (2×10^8 - 2×10^9 cfu/ml) to 9 ml of a concentrated biocide solution. After a 1 minute contact time, 1 ml of this mixture was added to 9 ml of the neutraliser to quench the effect of the biocide. Counting was then performed by the drop counting method.

A control to enable the calculation of ME was performed concurrently with each test, whereby biocide was replaced with SDW. The ME was then calculated as explained above.

2.11.7 Surface Disinfection Test

Surface disinfection tests were carried out to establish the efficacy of chlorhexidine against surface dried bacteria (Thomas *et al*, 2005).

Ten µl of washed bacterial culture was added to the bottom of a 28ml flat-bottomed glass bottle and left to dry at room temperature for 2 and 24 hours. After drying, 0.1ml of chlorhexidine (100 mg/L) was placed over the top of the dried cells and left for 5 minutes contact time. Neutraliser solution (0.9ml) was then added to quench the chlorhexidine, and a sterile magnetic stirrer used to resuspend the cells for 5 minutes.

The cells were then vortexed and counted using the drop counting method as described. The number of survivors following exposure to chlorhexidine was compared with those for the controls and the ME was calculated as described.

To enable the calculation of ME, a control was performed for each sample. Initially a non-drying control was used, whereby bacterial cells were added and without drying, SDW (replacing biocide) and neutraliser were added as per the above experimental details, and drop counting performed. The 2 and 24 hour dried tubes were inoculated at the same time and then left to dry.

Drying controls were also performed alongside these experiments, to attempt to take into account the effect of drying on the viability of the bacterial cells. Bacterial cells were dried concurrently with the tests for the same lengths of time, and treated as in the experiments, with SDW added in place of biocide. This would arguably give a more accurate ME as it would take into account the reduced viability of the bacterial cells after drying time.

2.11.8 Biocide Residue Test

This test examined the effect of chlorhexidine digluconate residues on bacterial isolates (Thomas *et al*, 2005). Stock solutions of chlorhexidine were prepared to give final concentrations of 25 mg/L. One ml was dispensed onto the bottom of a flat-bottomed glass bottle, the excess removed, and the bottles left to dry at room temperature for 2, 24

and 48 hours. After drying, 20µl of an overnight culture (1-2 x 10^8 cfu/ml) was added to the bottles containing the chlorhexidine residue. After 5 minutes contact time at room temperature, 1ml of neutraliser was added and an aliquot the counted using the previously described drop-counting method. Controls were performed concurrently with the tests to enable calculation of the ME. 20µl of the bacterial culture used in the corresponding test was added to 1 ml of neutraliser and counting performed using the drop counting method.

MICs of the exposed cells were also determined against a panel of antibiotics to examine the antibiotic susceptibility profiles after exposure to chlorhexidine.

Chapter 3: Characterisation of *Acinetobacter* isolates

3.1 Introduction

As discussed in Section 1.3, whilst *A. baumannii* is considered the most prevalent in clinical infections it is thought that other species, especially those of the Acb complex (gen.sp. 13TU and 3), are also relevant in the clinical environment (Dijkshoorn *et al*, 2007). Other species not frequently associated with infections are also often isolated. Additionally, sensitive clinical *A. baumannii* are not often isolated from the clinical environment as most are now multi-drug resistant. As such this study included a range of *Acinetobacter* spp clinical isolates from the hospital population with varying sensitivities.

The *Acinetobacter* genus is genotypically heterogenous and has a complex taxonomic history (as discussed in Section 1.3) hence speciation has traditionally been a problematic area. Edinburgh isolates collected for this study were typed in the RIE clinical labs using the Vitek system but, as discussed in Section 1.3.2, this is not an adequate method for speciation of *Acinetobacter*. As such the aforementioned (Section 1.3.2) tDNA fingerprinting method, detailed in Section 2.6.1.1, was used in the first instance to identify species.

However tDNA fingerprinting does not discriminate well between *A. baumannii* and gen.sp. 13TU, and as such PCR to detect $bla_{\text{OXA-51-like}}$ genes (Turton *et al*, 2006), as

detailed in Section 2.6.1.2 and discussed in Section 1.3.2, was used to distinguish between *A. baumannii* and gen.sp. 13TU. Additionally, several of these isolates have been typed by colleagues (Ben Evans and Dr Ahmed Hamouda, personal communication) using the 16S-23S rRNA intergenic spacer restriction analysis method (Section 1.3.2; Chang *et al*, 2005), which has enabled greater distinction between representative species.

There is evidence of inter-hospital circulation of several widespread outbreak strains of *A. baumannii*, the most prevalent in the UK being the OXA-23 clone 1 (Section 1.3.4; Coehlo *et al*, 2004). Several of these outbreak strains, received from Dr Jane Turton, are included as comparison in later experiments in this study and their sensitivities are analysed below.

3.2 Results

Acinetobacter spp isolates were collected in 2002 from the blood culture collection, Royal Infirmary Edinburgh, comprising infection-associated isolates from 1995-1999. After plating on MacConkey agar to isolate pure cultures, isolates were grown overnight in cryogenic vials containing nutrient broth, then stored at -70˚C in glycerol for further use. Isolates from previous worldwide collections, including from Argentina, Singapore and USA, were also included. Species strains used for comparison in tDNA speciation were kindly gifted by Dr Kevin Towner, and several prevalent outbreak strains were

kindly gifted by Jane Turton (strains detailed in Section 2.1) and were used as comparisons for several further experiments.

3.2.1 Minimum Inhibitory Concentrations

MICs were performed by the agar dilution method according to BSAC guidelines, as detailed in Section 2.7. MIC values of the tested antibiotics against the Edinburgh and selected worldwide isolates are detailed in Table 3.1. Recommended BSAC breakpoints (Andrews, 2001; MacGowan & Wise, 2001) were used to establish the sensitivity of the isolates, based on the MICs of the tested antibiotics.

Table 3.1 MICs of a range of antibiotics on the *Acinetobacter* spp. isolate collection

Isolates		Minimum Inhibitory Concentrations (mg/L)							
		AMP	**AUG**	**CTX**	**CAZ**	**GENT**	**CIP**	**IMP**	**MERO**
E	**3**	64	32	32	16	0.5	8	0.25	2
E	**5**	16	16	8	16	1	8	0.25	2
E	**6**	0.25	0.25	0.5	16	0.25	0.5	0.06	0.06
E	**7**	8	16	8	2	1	0.12	0.12	0.12
E	**8**	16	4	4	8	2	0.25	0.12	0.25
E	**9**	4	4	4	2	0.25	0.06	0.06	0.12
E	**10**	4	8	8	2	1	0.12	0.12	0.25
E	**11**	16	8	16	8	1	0.5	0.12	0.25
E	**12**	2	4	4	2	0.25	0.12	0.12	0.12
E	**13**	16	4	16	4	0.5	0.5	0.12	0.25
E	**14**	16	16	16	8	1	0.5	0.12	0.25
E	**15**	1	0.5	4	4	0.25	0.06	0.06	0.12
E	**16**	16	16	16	8	0.5	0.5	0.12	0.25
E	**17**	32	8	16	8	0.5	0.25	0.12	0.25
E	**18**	32	32	32	8	1	8	0.25	1
E	**19**	32	32	32	16	1	8	0.25	1
E	**20**	8	4	64	64	0.12	0.12	0.06	0.25
E	**21**	0.5	0.5	2	4	0.25	0.032	0.12	0.06
E	**22**	64	32	32	16	1	8	0.25	1
E	**23**	32	16	16	8	0.25	0.12	0.12	0.25
E	**24**	64	32	32	16	1	8	0.25	2

Table 3.1 continued		**Minimum Inhibitory Concentrations (mg/L)**							
		AMP	**AUG**	**CTX**	**CAZ**	**GENT**	**CIP**	**IMP**	**MERO**
E	**25**	32	8	32	8	0.25	0.25	0.12	0.25
E	**26**	4	4	2	2	0.25	0.25	0.12	0.12
E	**27**	16	16	16	16	0.25	0.12	0.25	0.5
E	**28**	128	32	32	16	4	0.5	0.25	1
E	**29**	64	16	32	16	0.5	8	0.25	1
E	**30**	32	8	4	4	1	0.06	0.12	0.25
E	**31**	128	32	64	16	0.5	8	0.25	1
E	**32**	64	16	32	16	0.5	8	0.25	1
E	**33**	1	0.5	4	4	0.25	0.12	0.06	0.06
E	**34**	128	32	32	16	1	8	0.25	1
E	**35**	64	32	32	16	1	8	0.25	1
E	**36**	64	32	32	8	1	8	0.25	1
E	**37**	64	16	32	16	0.5	8	0.25	1
E	**38**	128	16	32	16	0.5	8	0.25	1
E	**39**	64	16	32	16	0.5	8	0.25	1
E	**40**	64	32	32	16	0.5	32	0.25	1
E	**41**	32	16	16	8	1	0.5	0.12	0.25
E	**42**	32	16	16	8	1	0.5	0.12	0.25
E	**43**	16	8	4	4	1	0.06	0.12	0.25
E	**44**	2	0.5	4	4	0.12	0.06	0.06	0.06
E	**45**	64	32	32	16	1	8	0.25	1
E	**46**	128	32	32	16	1	8	0.25	1
E	**47**	32	8	4	4	1	0.06	0.12	0.25
E	**48**	64	16	32	16	0.5	8	0.25	1
E	**49**	64	32	32	16	1	32	0.5	1
E	**50**	64	32	32	16	1	32	0.25	1
E	**51**	64	32	16	16	1	32	0.25	1
U	**1**	>128	128	64	32	64	>128	0.25	0.5
U	**4**	16	16	16	8	2	2	0.06	0.25
U	**5**	32	16	32	16	1	0.25	0.12	0.5
U	**6**	32	16	32	16	2	>128	0.25	0.5
U	**7**	>128	>128	>128	>128	16	>128	2	8
U	**8**	1	1	2	2	0.06	0.03	0.03	0.06
U	**9**	>128	>128	>128	>128	32	128	0.25	1
U	**10**	64	32	64	16	0.25	1	0.12	0.5
U	**11**	>128	>128	>128	>128	16	128	1	8
U	**12**	16	16	16	8	0.25	0.5	0.12	0.25
U	**13**	>128	64	32	8	128	8	0.5	0.5
U	**14**	16	8	8	4	0.5	0.5	0.008	0.25
U	**15**	32	16	16	8	0.25	0.25	0.12	0.5
U	**17**	32	16	16	16	8	4	0.12	1
U	**20**	32	16	16	8	1	0.5	0.008	0.25
U	**21**	16	8	16	16	8	>128	0.25	0.5
U	**22**	>128	64	32	8	128	8	0.25	0.5
U	**23**	8	4	16	8	0.5	0.5	0.12	0.5
U	**24**	>128	64	16	8	128	16	0.25	0.5
U	**25**	32	16	16	8	0.25	0.25	0.12	0.25

Table 3.1 continued		**Minimum Inhibitory Concentrations (mg/L)**							
		AMP	**AUG**	**CTX**	**CAZ**	**GENT**	**CIP**	**IMP**	**MERO**
U	**27**	32	16	32	16	0.5	0.5	0.12	0.5
U	**28**	32	32	32	16	0.25	0.5	0.12	0.5
U	**29**	32	16	16	8	1	0.25	0.12	0.25
U	**30**	32	16	16	8	1	0.25	0.12	0.5
U	**32**	>128	64	16	8	32	1	0.06	0.5
U	**34**	16	8	16	4	0.5	0.5	0.06	0.25
U	**35**	32	32	32	8	1	0.5	0.12	0.5
U	**36**	128	64	32	32	1	64	0.5	2
U	**38**	>128	>128	128	32	2	>128	0.25	2
U	**41**	16	32	32	16	2	128	0.12	0.5
U	**43**	16	8	32	4	1	0.25	0.12	0.25
U	**45**	>128	>128	128	>128	>128	128	0.5	1
U	**46**	32	32	32	8	1	0.25	0.12	0.25
U	**47**	>128	>128	>128	>128	64	128	1	8
U	**49**	16	32	64	16	0.5	1	0.008	0.5
U	**51**	16	8	32	4	0.5	0.25	0.06	0.25
U	**52**	64	32	32	16	0.5	0.25	0.12	0.5
U	**53**	32	8	32	8	0.25	0.5	0.12	0.25
U	**59**	32	32	32	8	0.5	0.03	0.008	0.5
U	**60**	32	32	32	8	0.5	0.5	0.12	0.25
U	**61**	>128	>128	32	4	1	0.25	0.12	4
U	**62**	16	32	8	4	0.5	0.25	0.12	0.25
U	**63**	32	32	16	8	0.25	0.5	0.06	0.25
U	**64**	>128	>128	64	32	>128	>128	0.25	0.5
U	**66**	1	1	2	4	0.12	0.03	0.03	0.12
U	**67**	>128	>128	>128	>128	>128	128	2	16
U	**68**	32	32	32	8	0.5	0.25	0.25	0.25
U	**69**	32	32	16	8	1	0.25	0.12	0.25
U	**71**	8	4	16	4	0.25	0.12	0.12	0.25
U	**74**	0.25	0.12	1	1	0.06	0.015	0.015	0.06
U	**75**	>128	>128	128	32	>128	64	0.5	1
U	**76**	>128	>128	128	32	>128	128	0.25	1
U	**77**	>128	>128	128	32	>128	128	0.25	1
U	**78**	16	32	16	8	0.25	0.25	0.12	0.25
U	**79**	32	32	64	8	0.25	0.5	0.25	0.5
U	**80**	8	8	8	2	0.25	0.25	0.06	0.25
U	**81**	32	32	32	16	0.5	0.25	0.25	0.25
U	**82**	8	32	16	8	0.25	0.25	0.06	0.25
U	**83**	16	16	16	8	0.25	0.25	0.25	0.25
U	**84**	16	16	16	16	0.25	0.5	0.06	0.25
U	**86**	64	16	64	16	1	1	0.06	1
U	**87**	128	64	128	64	2	>128	1	2
U	**88**	16	16	16	8	0.5	0.12	0.06	0.25
U	**89**	32	16	64	16	0.5	0.5	0.12	0.5
U	**90**	16	16	16	8	0.5	0.5	0.12	0.25
U	**91**	>128	>128	>128	>128	32	>128	0.25	2
U	**93**	16	16	32	8	0.5	0.25	0.12	0.25

Table 3.1 continued		Minimum Inhibitory Concentrations (mg/L)							
		AMP	**AUG**	**CTX**	**CAZ**	**GENT**	**CIP**	**IMP**	**MERO**
U	**97**	16	16	16	8	0.5	0.25	0.12	0.5
U	**98**	>128	128	64	16	>128	>128	0.25	1
U	**99**	32	32	8	4	1	0.25	0.12	0.25
U	**101**	32	16	16	8	1	0.25	0.06	0.25
W	**779**	>128	128	64	4	0.5	>16	8	4
W	**783**	>128	>128	32	4	1	>16	16	8
W	**789**	>128	32	>128	>128	>64	>16	0.25	0.5
W	**790**	>128	>128	64	128	>64	>16	16	4
W	**868**	>128	>128	>128	>128	>128	>16	16	128

E – Edinburgh, U – USA, W – worldwide.

AMP - ampicillin, AUG – co-amoxyclavulanic acid, CTX – cefotaxime, CAZ – ceftazidime, GENT – gentamicin, CIP – ciprofloxacin, IMP – imipenem, MERO – meropenem.

Of the 128 isolates, the majority were susceptible to both imipenem (breakpoint > 4mg/L) and meropenem (breakpoint > 4mg/L), as would be expected, with 94% and 91% susceptible respectively. There was a high percentage resistance within the whole sample to both cefotaxime (breakpoint > 1mg/L) and ceftazidime (breakpoint > 2mg/L), at 98% and 94% resistance respectively. The majority of isolates were resistant or intermediate-resistant to ampicillin (breakpoint > 8mg/L; 66% resistant and 20% intermediate-resistant) and co-amoxiclavulanic acid (breakpoint > 8mg/L; 50% resistant and 27% intermediately resistant). There was 77% susceptibility to gentamicin (breakpoint > 4mg/L) and 54% susceptibility to ciprofloxacin (breakpoint > 1mg/L), with 3% of the isolates intermediate-resistant to ciprofloxacin.

Of the Edinburgh isolates (N=48) there was 100% susceptibility to both imipenem and meropenem, and 98% susceptibility to gentamicin. Similar to that described for the worldwide sample as a whole, there were high percentages of resistance to cefotaxime

and ceftazidime, of 98% and 90% resistance respectively. There was slightly more susceptibility to co-amoxiclavulanic acid, with 38% susceptibility in the Edinburgh isolates compared to 23% in the worldwide sample as a whole. There was also a slightly higher percentage susceptibility observed to ampicillin than in the worldwide sample, with 23% susceptibility in the Edinburgh isolates compared to 14% in the worldwide isolates. These figures are summarised in Table 3.2.

Table 3.2 Percentage sensitivities of the *Acinetobacter* spp. sample

Worldwide sample (N = 128)*								
	AMP	**AUG**	**CTX**	**CAZ**	**GENT**	**CIP**	**IMP**	**MERO**
% susceptible	14	23	2	6	77	54	94	91
% intermediate	20	27	0	0	1	3	0	0
% resistant	66	50	98	94	22	43	6	9
Edinburgh population (N = 48)								
	AMP	**AUG**	**CTX**	**CAZ**	**GENT**	**CIP**	**IMP**	**MERO**
% susceptible	23	38	2	10	98	54	100	100
% intermediate	17	29	0	0	2	0	0	0
% resistant	60	33	98	90	0	46	0	0

Breakpoints in brackets.
AMP – ampicillin (8mg/L), AUG – co-amoxyclavulanic acid (8mg/L), CTX – cefotaxime (1mg/L), CAZ – ceftazidime (2mg/L), GENT – gentamicin (4mg/L), CIP – ciprofloxacin (1mg/L), IMP – imipenem (4mg/L), MERO – meropenem (4mg/L).
* worldwide sample includes the Edinburgh isolates.

Based on their sensitivity profiles to all tested antibiotics, 22 representative resistant (N=8), intermediate (N=7) and sensitive (N=7) isolates were selected for further investigation. These isolates, their origin and sensitivities, are detailed in Table 3.3.

Table 3.3 Representative *Acinetobacter* spp. isolates used for further investigation

Isolate Information			MICs (mg/L)							
			AMP	AUG	CTX	CAZ	GENT	CIP	IMP	MERO
E9	Edinburgh	S	4	4	4	2	0.25	0.06	0.06	0.12
E10	Edinburgh	S	4	8	8	2	1	0.12	0.12	0.25
E13	Edinburgh	I	16	4	16	4	0.5	0.5	0.12	0.25
E14	Edinburgh	I	16	16	16	8	1	0.5	0.12	0.25
E15	Edinburgh	S	1	0.5	4	4	0.25	0.06	0.06	0.12
E21	Edinburgh	S	0.5	0.5	2	4	0.25	0.032	0.12	0.06
E26	Edinburgh	S	4	4	2	2	0.25	0.25	0.12	0.12
E33	Edinburgh	S	1	0.5	4	4	0.25	0.12	0.06	0.06
E41	Edinburgh	I	32	16	16	8	1	0.5	0.12	0.25
E51	Edinburgh	R	64	32	16	16	1	32	0.25	1
U7	USA	R	>128	>128	>128	>128	16	>128	2	8
U43	USA	I	16	8	32	4	1	0.25	0.12	0.25
U45	USA	R	>128	>128	>128	128	>128	128	0.5	1
U51	USA	I	16	8	32	4	0.5	0.5	0.06	0.25
U66	USA	S	1	1	2	4	0.12	0.03	0.03	0.25
U71	USA	I	8	4	16	4	0.25	0.12	0.12	0.25
U80	USA	I	8	8	8	2	0.25	0.25	0.06	0.25
W779	Argentina	MR	>128	>128	64	4	0.5	>128	8	4
W783	Argentina	MR	>128	>128	32	4	1	>128	16	8
W789	Argentina	MR	>128	32	>128	>128	>64	>128	0.25	0.5
W790	Argentina	MR	>128	>128	64	128	>64	>128	16	4
W868	Singapore	MR	>128	>128	>128	>128	>128	>128	16	128

S – Sensitive, I – Intermediate, R – Resistant, MR – Multi-resistant.

MICs were also performed on the prevalent *A. baumannii* outbreak strains, gifted by Dr Jane Turton, which were included in several later experiments. These strains and their sensitivity profiles are detailed in Table 3.4.

Table 3.4 Details of outbreak strains used in further experiments

		MICs (mg/L)					
Isolate	Description	AMP	CTX	CAZ	GENT	CIP	MERO
JTA	NW strain	>128	>128	>128	4	0.032	4
JTB	W strain / European clone 1	8	32	0.016	0.016	0.016	0.12
JTC	Midlands 2	>128	>128	128	>128	128	2
JT3	T strain	>128	>128	128	32	128	1
JT4	SE Clone	>128	>128	128	16	0.016	8
JT6	OXA-23 Clone 2	>128	>128	>128	64	64	32

3.2.2 Speciation of Representative Isolates

tDNA fingerprinting (Section 2.6.1.1) was performed on the representative isolates detailed in Table 3.2, as described in Section 2.6.1.1. The BioNumerics™ programme (Applied Maths, Ghent, Belgium) was used to store and analyse resultant band patterns by comparison with standard strains. This software devises algorithms to enable analysis of isolate profiles. Here the Dice coefficient and cluster analysis using hierarchical un-weighted pair arithmetic average algorithm (optimisation 1.5%, tolerance 1%) were used. Comparisons are displayed in Figures 3.1 – 3.3.

A comparison of the tDNA band patterns of the representative isolates (Figure 3.1) shows varied patterns within the population, although many had a close match with the ATCC 19606 (*A. baumannii*) or gen. sp. 13TU species patterns. These are shown in Figure 3.2, whilst non-*baummannii* /gen.sp. 13TU patterns are shown in Figure 3.3.

Figure 3.1 tDNA band patterns of representative isolates

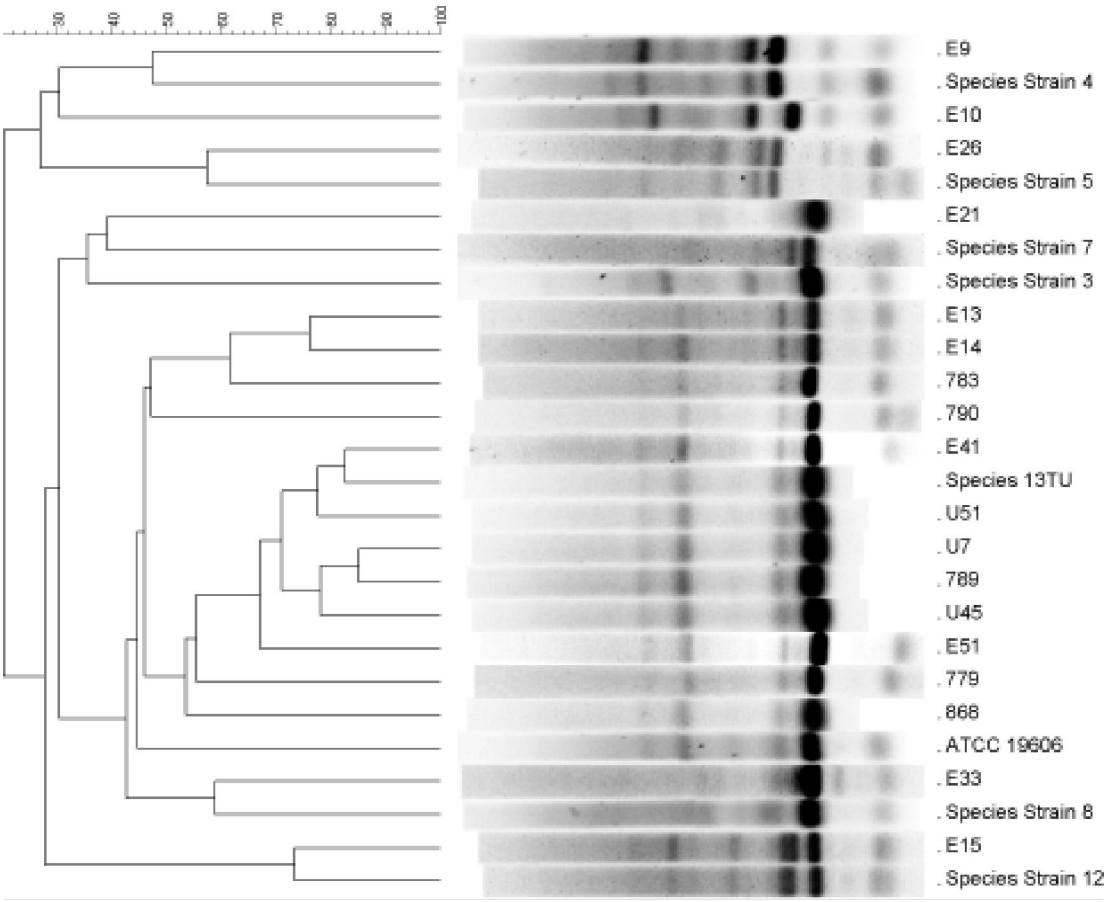


Figure 3.2 tDNA band patterns of suspected *A. baumannii* or gen.sp. 13TU isolates from representatives

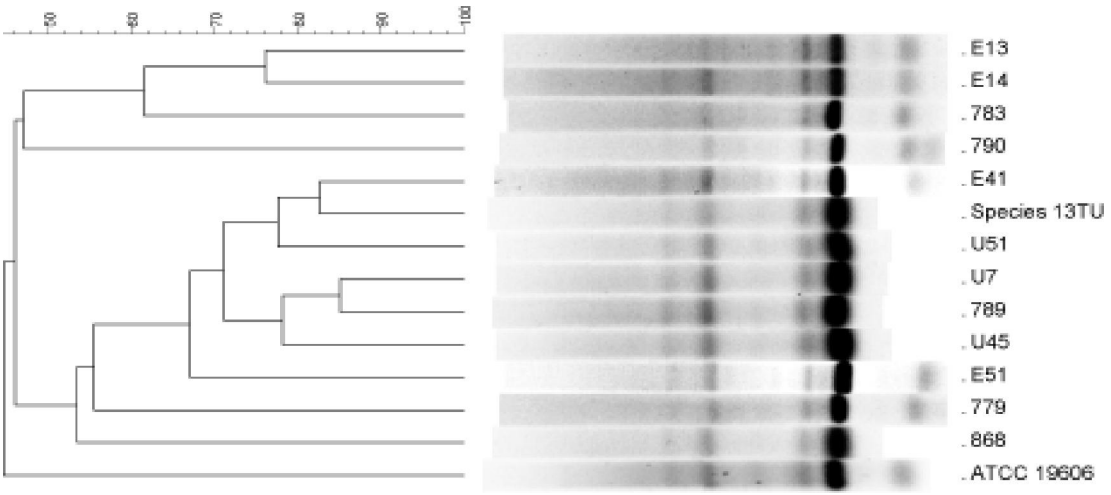
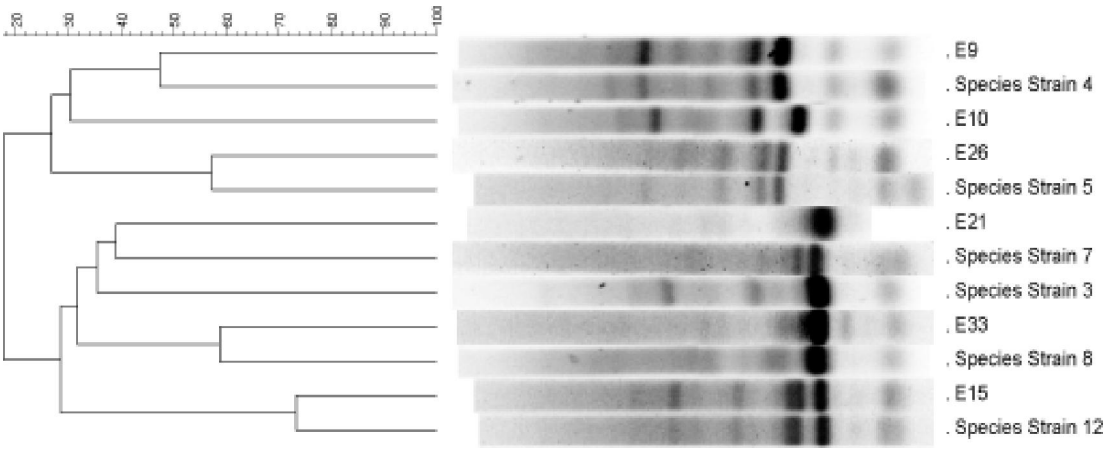


Figure 3.3 tDNA band patterns of other representative isolates not resembling *A.baumannii* or gen.sp. 13TU



Of those that did not have close similarity to the *A. baumannii*/gen.sp. 13TU band pattern, there were several that had a similarity to the pattern of other species strains; Isolate E15 had a 70% similarity to species strain 12 (*A. radioresistens*), isolate E26 had a >55% similarity to species strain 5 (*A. junii*) and isolate E33 had a >55% similarity to species strain 8 (*A. johnsonnii*). Several isolates (E9, E10 and E21) did not match to any significance any band pattern of the species strains tested. Isolate E9 had a <50% similarity to species strain 4 (*A. haemolyticus*), E10 clustered with 3 other Edinburgh clinical isolates (>40% similarity) and E21 had some similarity (<40%) to species strain 7 (*A. johnsonnii*).

PCR for the presence of $bla_{\text{OXA-51-like}}$ genes was also performed, as detailed in Section 2.6.1.2, the results shown in Table 3.5. Of the representative isolates which did have the *A. baumannii*/gen.sp. 13TU tDNA band pattern, several were subsequently found not to have the OXA-51 gene and were therefore most likely species 13TU.

Additionally, the isolates have since been included in further studies by Dr Ahmed Hamouda and Ben Evans in the Molecular Chemotherapy lab (personal communication), and have been typed to a certain extent by 16S-23S intergenic rRNA restriction (Chang *et al*, 2005). The results from these methods are compiled in Table 3.5, with conclusions as to the species of the isolates.

Table 3.5 Speciation results for the representative *Acinetobacter* spp isolates

Isolate		tDNA	OXA-51-like	16S-23S intergenic rRNA restriction*	Conclusion
E9	S	(*A. haemolyticus*?)	-	3,13TU	gen.sp.3
E10	S	?	-	3,13TU	gen.sp.3
E13	I	*A. baumannii* /13TU	+	2	*A. baumannii*
E14	I	*A. baumannii* /13TU	-	13TU	13TU
E15	S	*A. radioresistens*	-	13TU?	*A. radioresistens*
E21	S	*(A. johnsonii)?*	-	-	*A. johnsonii*
E26	S	*A. junii*	-	?	*A. junii*
E33	S	*A. johnsonii*	-	?	*A. johnsonii*
E41	I	*A. baumannii* /13TU	-	13TU?	13TU
E51	R	*A. baumannii* /13TU	-	13TU?	13TU
U7	R	*A. baumannii* /13TU	+	2	*A. baumannii*
U43	I	?	-	13TU	13TU
U45	R	*A. baumannii* /13TU	+	2	*A. baumannii*
U51	I	*A. baumannii* /13TU	+	2	*A. baumannii*
U66	S	?	+	2	*A. baumannii*
U71	I	?	+	2	*A. baumannii*
U80	I	?	-	3	3

* Ben Evans, personal communication.
S - sensitive, I – intermediate, R – Resistant.

The multi-resistant isolates, from Argentina and Singapore, had been speciated as *A.baumannii* in a previous study by DNA-DNA hybridisation (Dr Susan Brown, personal communication), and were all confirmed as such by possession of the *A.baumannii*/gen.sp. 13TU tDNA band pattern, and the presence of $bla_{\text{OXA-51-like}}$ (Figure 3.1).

The U isolates, a collection from the USA, had previously been typed and those included had been determined to be *A. baumannii* (Higgins, 2002). It was apparent (Table 3.5) that in fact several of these were not *A. baumannii* isolates; U43 and U80 did not possess $bla_{\text{OXA-51-like}}$ and were identified by 16S-23S intergenic rRNA restriction to be of gen.sp. 13TU and gen.sp. 3 respectively.

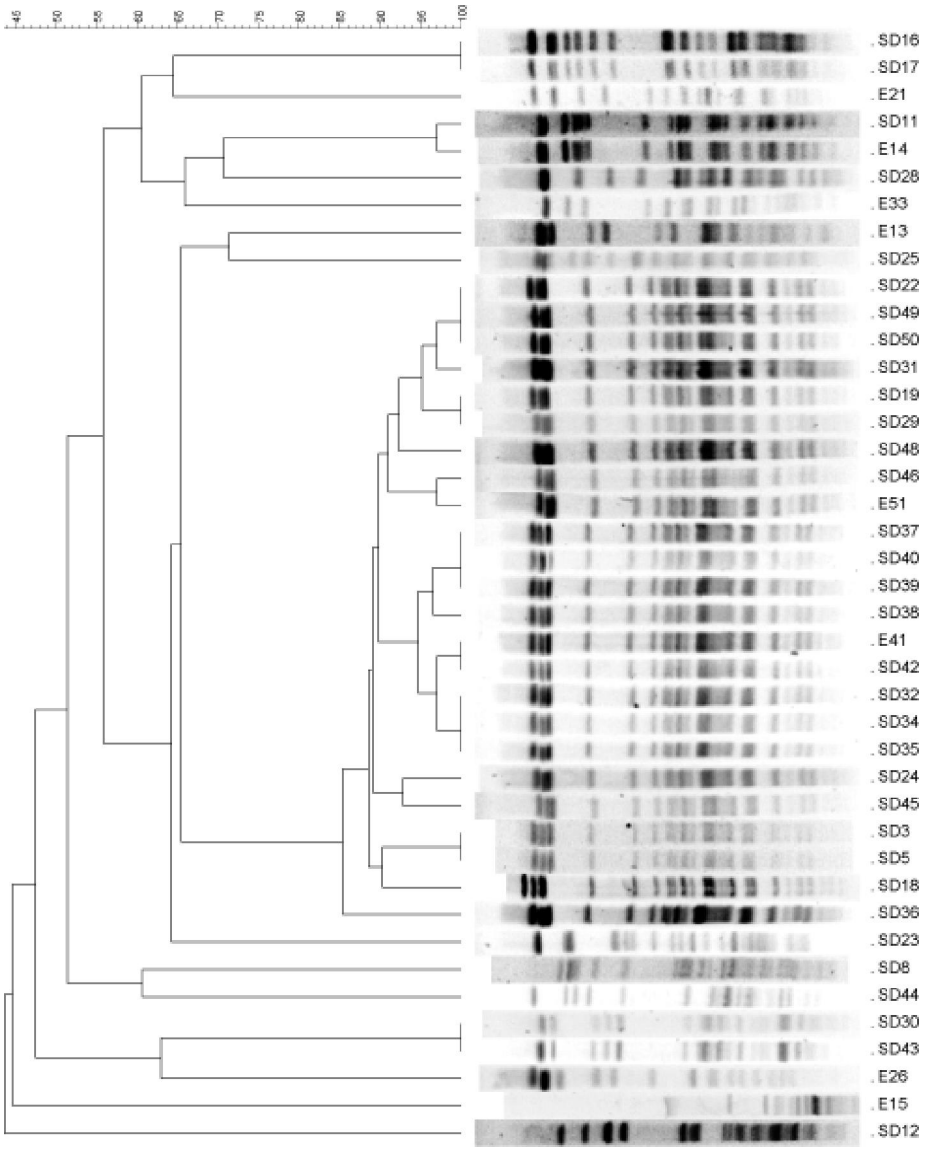
Taken together, within the limits of these methods, the representative isolates comprised: 8 resistant isolates of which 7 were *A. baumannii* and 1 was gen.sp. 13TU; 7 intermediate isolates of which 3 were *A. baumannii*, 3 were gen.sp. 13TU and 1 was gen. sp. 3; and 7 sensitive isolates of which 1 was *A. baumannii*, 2 were probable gen.sp. 3, 2 were *A. johnsonii* , 1 was *A. radioresistens* and 1 was *A. junii*.

3.2.3 Typing of Representative Isolates

PFGE of *Apa*I digested DNA from the representative isolates was performed (Section 2.8) to establish their relatedness (Section 1.3.2). Figure 3.4 shows the *Apa*I digested PFGE patterns for all Edinburgh isolates.

Analysis of the PFGE results of *Apa*I digested DNA of all Edinburgh isolates (N=48) reveals several potential clones of *Acinetobacter* spp that appear to be present in the RIE, resistant to most of the tested antibiotics (Figure 3.4).

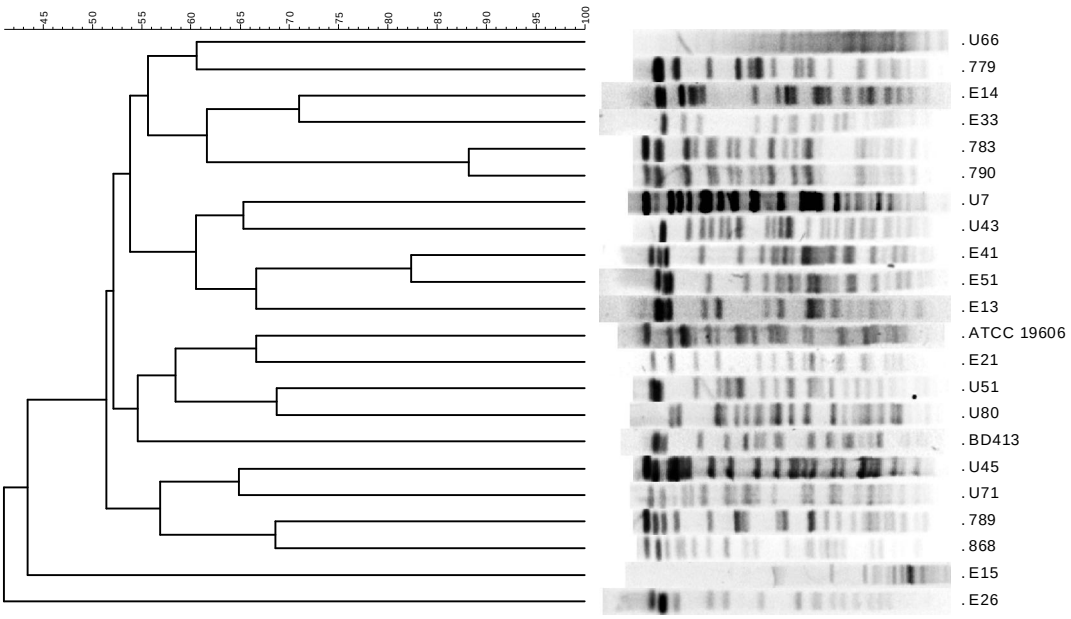
Figure 3.4 Dendrogram of *Apa*I digested PFGE patterns of Edinburgh isolates



SD and E both signify Edinburgh isolates, where E denotes those which are representative isolates.

From analysis of the PFGE results of *Apa*I-digested DNA it was apparent that the representative isolates were genotypically diverse (Figure 3.5). The most closely related were isolates 783 and 790 (88% similarity), both multi-resistant isolates from Argentina. Also closely related (82% similarity) were isolates E41 and E51, both from Edinburgh. E14 and E33, respectively intermediate and sensitive Edinburgh isolates, were 71% similar. Interestingly, clinical Edinburgh isolate E26 formed a separate branch to the rest of the representative isolates, whilst the non-clinical isolate ADP1 (shown on the diagram as BD413) was amongst the rest of the clinical isolates and was 66% similar to isolate E21.

Figure 3.5 Dendrogram of ApaI digested PFGE patterns of representative isolates



3.3 Discussion

Both the Edinburgh and worldwide sample had varied sensitivities to a range of antibiotics. The worldwide sample included several selected multi-resistant isolates (from Argentina and Singapore) which would have increased its overall resistance profile. However, generally the worldwide sample had similar percentage susceptibilities to the selected Edinburgh sample. There was notably more susceptibility to gentamicin, and marginally more susceptibility to imipenem and meropenem, in the Edinburgh sample.

Representative isolates were selected on the basis of their sensitivities to a range of antibiotics and were comprised of various *Acinetobacter* spp. The intermediate and resistant isolates were all of the clinically prevalent species *A. baumannii*, gen.sp. 13TU and gen.sp. 3. The sensitive isolates, however, were much more varied, including only one *A. baumannii* and two gen.sp. 3. This agrees with what is known of the general clinical population of *Acinetobacter* spp, as discussed in Section 1.3; that true *A. baumannii* with sensitive susceptibility profiles are now rarely isolated from the clinical environment, and that the most common and clinically important isolates are *A. baumannii* followed by gen.sp. 13TU and gen.sp. 3 (Section 1.3; Dijkshoorn *et al*, 2007; Dr Kevin Towner, personal communication).

This study aims to examine the development of antibiotic resistance in the clinical *Acinetobacter* population, compared to that present in already established resistant

isolates. It is therefore important to examine a sample of the clinical population, comprising *Acinetobacter* spp with varying sensitivities, to gain a valid insight into the hospital environment. Whilst *A. baumannii* is undoubtedly the most prevalent and important clinical species, it is clear from the data here that other species are also present and may have a role to play in the rise of resistance in the hospital population as a whole; *A. johnsonnii*, *A. lwoffii* and *A. radioresistens* have been reported as frequent colonisers of healthy human skin and there are rare cases of involvement in infections (Section 1.3.3; Seifert *et al*, 1993; Seifert *et al*, 1997; Berlau *et al*, 1999). Additionally, given the discussed difficulties in speciation of the *Acinetobacter* genus, it may be that some isolates reported as *A. baumannii* are actually different species, and their importance thus far has been overlooked. To better determine what may contribute to the success of *A. baumannii*, this study also examines other *Acinetobacter* spp isolated in the clinical environment. Indeed, the Edinburgh isolates examined here were all associated with infection, though this does not preclude their prior presence as colonisers.

The representative isolates generally were genotypically diverse, as determined by PFGE of *Apa*I restricted DNA. The most closely related isolates 783 and 790 were both multi-drug resistant isolates from Argentina. Edinburgh gen.sp. 13TU isolates E41 and E51 were also closely related. E41 was isolated in 1998 and was of an intermediate susceptibility, whilst E51 was isolated in 1999 and was resistant. It may be that these isolates represent an example of the progression of an intermediate isolate to a resistant isolate within the clinical environment.

It is apparent from the speciation data that the Vitek™ automated system, as used at the time in the Edinburgh Royal Infirmary clinical laboratory, is an inadequate method for speciating *Acinetobacter* spp. It has previously been reported that all species of the Acb complex are often identified by such systems as *A. baumannii*, and that many other species are not identified at all (Section 1.3.2; Bernards *et al*, 1996; Apisarnthanarak *et al*, 2007; Zbinden *et al*, 2007), and the above results seem to support this. They also show that it is essential when analysing other studies to examine the speciation methods used by the authors, especially when the exact species is of greater relevance; it may be that isolates previously identified as *A. baumannii* are not true *A. baumannii* species as identified by current methods.

Speciation of *Acinetobacter* spp is a continual problem. Whilst recent molecular techniques are more effective at delineating species, the 'gold standard' method of DNA-DNA hybridisation is not readily available to the average clinical or microbiology laboratory. There is still a lack of a universally comparable, rapid method that can be used routinely in these laboratories; this has traditionally limited our ability to examine and understand the epidemiology of clinical *Acinetobacter* spp and may have led to important interactions between clinical species being overlooked. Here the best methods available in the standard microbiological laboratory have been used to speciate the representative isolates.

Chapter 4: The *mutS* gene of Clinical *Acinetobacter* spp Isolates

4.1 Introduction

The general assumption is that antibacterial resistance results from use and misuse of antibiotics, which leads to the selection of resistant strains from a sensitive population. Increasing evidence has led to the hypothesis of subpopulations of genetically distinct mutator cells, able to rapidly acquire resistance through defects in MMR.

As discussed in Section 1.5, mutation is a fundamental aspect of the development and evolution of bacterial antibiotic resistance. Hypermutation is increasingly being recognised as a potential factor in antibiotic resistance development; mutators, most commonly deficient in MMR through defective *mutS*, have been reported in natural populations of several bacteria and have been associated with increased antibiotic resistance. In addition hypermutation is considered a key factor in the development of resistance in *P.aeruginosa* (Section 1.5.3; LeClerc *et al*, 1996; Matic *et al*, 1997; Oliver *et al*, 2000; Miller *et al*, 2002; O'Neill & Chopra, 2002; Watson Jr. *et al*, 2004).

Whilst the importance of mutator strains has been studied extensively in several bacteria there has been little investigation to date of the *mutS* gene of clinical *Acinetobacter* spp. Young and Ornston, as mentioned in Section 1.5.5, characterised and investigated the role of *mutS* from *Acinetobacter* strain ADP1, which has since been found to belong to

the newly described species *Acinetobacter baylyi* (Young & Ornston, 2001; Vaneechoutte *et al*, 2006). It was found that strains lacking MutS function had increased spontaneous mutation frequencies to rifampin and significantly increased transformation frequencies for divergent donors (Young & Ornston, 2001). This suggests that there is potential that mutations in the *mutS* gene of clinically relevant *Acinetobacter* spp may be associated with hypermutation and the development of antibiotic resistance, similar to the situation found in other bacteria.

The aims here were: to study for the first time the *mutS* gene of clinical *Acinetobacter* spp. isolates with varying susceptibilities (the representative isolates detailed in Chapter 3); to determine whether the *mutS* gene of clinical isolates varied compared to non-clinical strains; to examine whether clinical isolates had varying *mutS* sequences and, if so, whether different *mutS* types were associated with different sensitivities and species; and, described in the subsequent chapter, to determine whether there was any correlation between different *mutS* types and the mutation potential of the isolates.

4.2 Results

4.2.1 Design and Use of Degenerate Primers

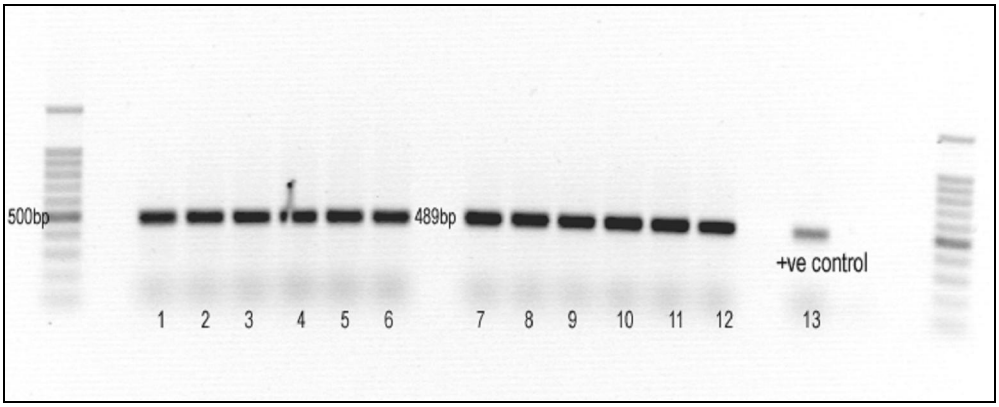
Young and Ornston (2001) amplified a 1.9kb section of the *mutS* gene using degenerate primers MutSf2 and MutSf3. However our repeated attempts at amplifying this large

section of the clinical isolates did not succeed, so degenerate primers (Section 2.3) were designed by Dr Susan Brown from areas of amino acid conservation in the *mutS* sequences of *Acinetobacter* sp. strains ADP1, 93A2, AD321, AC423D and LUH540 (sequences from Young and Ornston, 2001).

The first 180bp of the N-terminal region of MutS was examined, as this contains the mismatch recognition domain (aa 2-115 w.r.t. *E. coli*) (Lamers, 2000). The initial primer pair used to amplify the N-terminal region required lengthy optimisation of the PCR technique, and not all isolates were successfully amplified. This primer pair was eventually abandoned and primers LH1/C and RH1/F (sequences and methods detailed in Sections 2.3 and 2.9) were used, again after lengthy optimisation, to successfully amplify the N-terminal region of the *mutS* gene of all selected clinical isolates, with ADP1 and ATCC 19606 included as controls. The resultant PCR products were visualised on agarose gels (Figure 4.1) and the specific bands at 489 bp were excised, purified and sent for sequencing, as per the methods detailed in Section 2.9.

Consensus sequences for each isolate were derived from alignment of the received LH and RH primer nucleotide sequences, and the resultant consensus *mutS* sequences were used for comparisons. Sequences were compared from all the clinical isolates, within each sensitivity subset (i.e. sensitive, intermediate and resistant) and also to the non-clinical sequences (from Young & Ornston, 2001), by alignment using hierarchical clustering (Corpet, 1988).

Figure 4.1 Example of an agarose gel of *mutS* PCR products with bands of 489bp
Lanes 1-6: U51. Lanes 7-12: E14. Lane 13: ADP1.



4.2.2 Sequence Analysis – all Representative Isolates

Figure 4.2 shows the alignment of the amino acid sequences of the N-terminal region of *mutS* of all the examined clinical isolates, alongside the non-clinical strains. The controls (C) are at the top and the clinical isolates are organised into sensitivities: multi-resistant (MR), resistant (R), intermediate (I) and sensitive (S).

It was apparent that all examined clinical isolates had in common a section of five deleted amino acids, from aa 102-106 inclusive, compared to ADP1. This was also the case for one of the non-clinical strains (AC423D). Within the examined 180aa section of the MutS protein sequence there were 11 amino acid positions at which all of the clinical isolates varied compared to ADP1. At 7 of these positions the change compared to the sequence of ADP1 was conserved, whereas at the other four positions there were variations within the clinical isolates.

There were 39 positions within the same region of *mutS* at which there was amino acid variation in one or more of the clinical isolates, compared to the sequence of ADP1. At 12 positions the majority of the clinical isolates had a conserved change compared not only to ADP1, but also to the other non-clinical strains. These instances are highlighted in red in Figure 4.2. Of these 12 positions, there were three at which all clinical isolates had the same amino acid. At the other positions there was either no variation or an alternative variation in one or more of the clinical isolates; with the exception of aa 67, this occurred solely in the sensitive clinical isolates.

Figure 4.2 Alignment of the *mutS* gene of clinical isolates and non-clinical strains

Numbering w.r.t ADP1. Variations compared to ADP1 highlighted.
* indicates a position of variation of one or more of the clinical isolates compared to ADP1.
Red – highlights variation compared to ADP1 and the other non-clinical isolates.
Blue – highlights variation compared to ADP1, but same aa as one or more of the other non-clinical isolates.
Yellow – highlights an alternative variation where the majority of clinical isolates have a conserved variation.
Green – highlights non-conserved variations compared to ADP1, other non-clinical isolates and clinical isolates.
MR = multi-resistant, R = resistant isolates, I = intermediate isolates, S = sensitive isolates, C = controls.

```
                1                                                    **    *      60
ADP1    (C)     MNSTETMADL SSYTPMMQQY FKVKLEHQHA LLFYRMGDFY ELFFDDARKA AKFLGITLTH
93A2    (C)                           KVKLEHQHA LLFYRMGDFY ELFFDDARKA AKLLGITLTH
AD321   (C)                                                ELFFEDAHKA AKLLGITLTH
AC423D  (C)                                                ELFFEDAHKA AKLLGITLTH
ATCC19606                                                            AKLLGITLT-
779     (MR)                                               FEDAHLA AKLLGITLTH
783     (MR)                                              FFEDAHLA AKLLGITLTH
789     (MR)                                              FFEDAHLA AKLLGITLTH
790     (MR)                                                        LTH
868     (MR)                                                 AHLA AKLLGITLTH
U7       (R)                                                        H
U45     (R)                                                  AHLA AKLLGITLTH
E51     (R)                                                FEDAHLA AKLLGITLTH
E13     (I)                                               LFFEDAHLA AKLLGITLTH
E14     (I)                                               LFFEDAHLA AKLLGITLTH
E41     (I)                                               LFFEDAHLA AKLLGITLTH
U43     (I)                                               LFFEDAHLA AKLLGITLTH
U51     (I)                                               LFFEDAHLA AKLLGITLTH
U71     (I)                                               LFFEDAHLA AKLLGITLTH
U80     (I)                                               LFFEDAHLA AKLLGITLTH
E9      (S)                                              ELFFEDAHLA AKLLGITLTH
E10     (S)                                              ELFFEDAHLA AKLLGITLTH
E15     (S)                                                   AHKA AKLLGITLTH
E21     (S)                                                FEDAHKA AK-LGITLTH
E26     (S)                                               LFFEDAHLA AKLLGITLTH
E33     (S)                                                FEDAHKA A--IGITLTH
U66     (S)                                               LFFEDAHKA AKILGITLTH
```

Figure 4.2 continued

```
                61                                                                  120
                   ** *         * *          **  *  *    *              * ** *   *
ADP1    (C)     RGKANGEPIP MAGVPYHAAE GYLARLVRAG QTVAICEQVG EGENAGSRCK APMERKVVRI
93A2    (C)     RGKANGEPIP MAGVPYHAAE GYLARLVRAG QTVAICEQVG EGENAGSRGK APMERKVVRI
AD321   (C)     RGKANGEPIP MAGVPYHAAE GYLARLVKAG QTVAICEQVG EGESAGSRGK APMERKVVRI
AC423D  (C)     RGKANGEPIP MAGVPYHAAE GYLARLVKKG ETVVICEQIG E-----VTGK APVERGVVRI
ATCC19606       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVV-I
779     (MR)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
783     (MR)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
789     (MR)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
790     (MR)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
868     (MR)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U7      (R)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U45     (R)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E51     (R)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E13     (I)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E14     (I)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E41     (I)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U43     (I)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U51     (I)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U71     (I)     RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U80     (I)     RGKASGRPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E9      (S)     RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E10     (S)     RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E15     (S)     RGKANGTPIP MAGVPYHSAE GYLARLVKAG ETVAICEQVG E-----VTGK GPVDRQVVRI
E21     (S)     RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
E26     (S)     RGKTNGEPIP MAGVPYHSAE GYLARLVKSG RTVAICEQVG E-----VTGK GPVERKVVRV
E33     (S)     RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
U66     (S)     RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI

                121                                                                 180
                *    *       *** *     *  ** ***  *     *         **** **  *
ADP1    (C)     ITPGTITDDA LLGSYQSSNL VALCIQQNKI GIALLDLSAS IFKVQQHEFK TEQLYIELAR
93A2    (C)     ITPGTITDDA LLGSYQSSNL VALCIQQNKI GIALLDLSAS IFKVQQHEFK TEQLYIELAR
AD321   (C)     ITPGTITDDA LLGSYQSSNL VALCIQQNKI GLALLDLSAS IFKVQEHDFK TEQLAIELSR
AC423D  (C)     ITPGTLTDDA LLTAHQSSNL VALCVQQNEI GIALLDLSAG LFKVQQQEFQ LEQLGIELSR
ATCC19606       LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLS-G IFKVQQQDYK PEQLPIELAR
779     (MR)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
783     (MR)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
789     (MR)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
790     (MR)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
868     (MR)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U7      (R)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U45     (R)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E51     (R)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E13     (I)     LTPGTLTEDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E14     (I)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E41     (I)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U43     (I)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U51     (I)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U71     (I)     LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U80     (I)     LTPGTLTDDA LLSSYQSSNL VSLCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E9      (S)     LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E10     (S)     LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E15     (S)     LTPGTLTD-A LLSSHQSSNL VALCFQQNQV GIALLDLGAG IFKVQQQDYK PEQLTIELAR
E21     (S)     ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
E26     (S)     LTPGTLTDDA LLSSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQTYK PEQLPIELAR
E33     (S)     ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
U66     (S)     ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
```

At 16 positions one or more of the clinical isolates had a change compared to the *mutS* sequence of ADP1 but the amino acid matched one or more of the other non-clinical isolates at this position. These instances are highlighted in blue in Figure 4.2, where the equivalent amino acid in the non-clinical strains is also highlighted. At four of these 16 positions the change was observed in all clinical isolates and was conserved throughout. At nine of the 16 positions a conserved change was seen only in one or more of the sensitive isolates. At the further three positions there was either a variation in the change seen or no change compared to ADP1, in one or more of the clinical isolates.

There were 15 instances at five positions within this section of the *mutS* sequence where there was a conserved change compared to ADP1 in the majority of clinical isolates but variation in one or more of them. These instances are highlighted in yellow in Figure 4.2. Most of these instances occurred in the sensitive isolates, but there were two which occurred in intermediate isolate U80 (at aa 67 and 133).

Within the examined 180aa section there were a further 30 instances, at 16 positions, where there was non-conserved amino acid variation compared to the sequences of all the non-clinical isolates and to the other clinical isolates. These instances are highlighted in green in Figure 4.2. Again these instances occurred mostly within the sensitive isolates, but also at two positions in U80 (aa 65 and 142). At seven of the 16 positions, the amino acid was conserved in two or more of the sensitive isolates.

To summarise, in comparison to ADP1 there was substantial variation in the clinical isolates. Some of these aa variations were conserved in most clinical isolates and differed also to the other non-clinical controls (highlighted in red in Figure 4.2). At other positions of variation compared to ADP1, there were similarities in the clinical isolates with one or more of the non-clinical controls (highlighted in blue in Figure 4.2). Additionally, variation between the clinical isolates was apparent, either at positions where most clinical isolates varied compared to ADP1, or in positions where few clinical isolates varied compared to ADP1 (highlighted in yellow and green respectively in Figure 4.2).

Figure 4.3 shows the *mutS* amino acid sequence alignment of all the clinical isolates, to enable observation of differences within the clinical population. For clarity, only the main section of the amplified N-terminal region is shown (aa 61-180 wrt ADP1), as this was the area in which the variations within the clinical isolates were observed. As the numbering is wrt ADP1 amino acids 102-106 inclusive are blank as this was an area of deletion common to all the examined clinical isolates.

Highlighted in red in Figure 4.3 are 87 instances at 23 positions where there was conserved amino acid variation within two or more of the clinical isolates, compared to the majority of the clinical isolates (compared to the clinical isolates' consensus sequence). There were two positions (aa 65 and 133) where this occurred in intermediate isolate U80, but the majority of instances were within the sensitive *Acinetobacter* spp isolates.

Figure 4.3 Alignment of the amino acid sequence of the *mutS* of clinical isolates

Numbering w.r.t. ADP1. Variations highlighted.
* indicates a position at which there is amino acid variation in one or more isolates compared to the clinical isolates' consensus.
Red = conserved variation within the clinical isolates.
Blue = non-conserved variation within the clinical isolates.

```
          61                                                          120
             ** *         * *           *  *  *    *                * *   *
779 (MR) RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
783 (MR) RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
789 (MR) RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
790 (MR) RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
868 (MR) RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U7  (R)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U45 (R)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E51 (R)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E13 (I)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E14 (I)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E41 (I)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U43 (I)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U51 (I)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U71 (I)  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U80 (I)  RGKASGRPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E9  (S)  RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E10 (S)  RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E15 (S)  RGKANGTPIP MAGVPYHSAE GYLARLVKAG ETVAICEQVG E-----VTGK GPVDRQVVRI
E21 (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
E26 (S)  RGKTNGEPIP MAGVPYHSAE GYLARLVKSG RTVAICEQVG E-----VTGK GPVERKVVRV
E33 (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
U66 (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI

          121                                                         180
          *           *** *     *  ** * *  *     *   *     ** * **  *
779 (MR) LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
783 (MR) LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
789 (MR) LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
790 (MR) LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
868 (MR) LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U7  (R)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U45 (R)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E51 (R)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E13 (I)  LTPGTLTEDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E14 (I)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E41 (I)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U43 (I)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U51 (I)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U71 (I)  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U80 (I)  LTPGTLTDDA LLSSYQSSNL VSLCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E9  (S)  LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E10 (S)  LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E15 (S)  LTPGTLTD-A LLSSHQSSNL VALCFQQNQV GIALLDLGAG IFKVQQQDYK PEQLTIELAR
E21 (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
E26 (S)  LTPGTLTDDA LLSSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQTYK PEQLPIELAR
E33 (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
U66 (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
```

There were a further 14 instances, at 12 positions, where there was non-conserved amino acid variation in a clinical isolate compared to the clinical isolates' consensus sequence. Again the majority of these instances occurred only within the sensitive isolates, with two exceptions occurring in intermediate isolate U80 (*Acinetobacter* gen.sp. 3) at amino acids 67 and 142.

From the sequences shown in Figure 4.2 it appears that the resistant, multi-resistant, and most of the intermediate clinical isolates have a highly conserved MutS sequence within this 180aa region. Instances of amino acid variation in this region of the MutS protein appeared to be confined to the sensitive *Acinetobacter* spp isolates, with a few exceptions as noted.

4.2.3 Sequence Analysis – Resistant isolates

From Figure 4.3 above, it was established that the amino acid sequence of the multi-resistant and resistant isolates was highly conserved. A comparison between the resistant isolates and the non-clinical control ADP1, along with the *A. baumannii* type strain ATCC 19606 is shown in Figure 4.4. This allows for observation of any amino acid changes in the highly conserved resistant isolates' sequences compared to the non-clinical ADP1 strain.

Figure 4.4 Alignment of the amino acid sequence of the *mutS* of resistant and multi-resistant clinical isolates with ADP1 and ATCC 19606

Numbering wrt ADP1.
* indicates position at which there is amino acid variation compared to ADP1.
Red = conserved amino acid change compared to ADP1.

```
           61                                                        120
                 *          *          *   *                ***    *
ADP1       RGKANGEPIP MAGVPYHAAE GYLARLVRAG QTVAICEQVG EGENAGSRCK APMERKVVRI
ATCC19606  RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVV-I
779 (MR)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
783 (MR)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
789 (MR)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E51 (R)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U45 (R)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
868 (MR)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U7  (R)    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
790 (MR)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI

           121                                                       180
           *    *       *             *  *   *       *       ***  *   *
ADP1       ITPGTITDDA LLGSYQSSNL VALCIQQNKI GIALLDLSAS IFKVQQHEFK TEQLYIELAR
ATCC19606  LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLS-G IFKVQQQDYK PEQLPIELAR
779 (MR)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
783 (MR)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
789 (MR)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E51 (R)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U45 (R)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
868 (MR)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U7  (R)    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
790 (MR)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
```

The amino acid sequence of ATCC 19606 was identical to that of the resistant and multi-resistant clinical isolates. There were 20 positions within this region (marked in Figure 4.4) at which there were identical amino acid variations in the resistant clinical isolates compared to ADP1. These changes are also detailed in Table 4.1. This number does not include the aforementioned 5 deleted amino acids. There were no occurrences of non-conserved amino acid variation within the resistant and multi-resistant isolates; within the isolates examined this region of the *mutS* gene was highly conserved.

To the best of the author's knowledge this highly conserved sequence comprising novel amino acid changes compared to ADP1 is unreported to date, and has been submitted in the course of this study into Genbank (Genbank accession number for partial *mutS* sequence: DQ 989864). Subsequently this *mutS* sequence is referred to as the R-type sequence, for ease of comparison with the other clinical isolates.

There was no amino acid variation within the resistant isolates (comprised of *A.baumannii* and gen.sp. 13TU) and from analysis of the nucleotides at the positions where there was amino acid variation in the resistant isolates compared to ADP1 it was found that there were only 5 instances of nucleotide variation at these positions (detailed in Table 4.1).

Further analysis of the nucleotide sequences of the resistant clinical isolates showed that they were a highly conserved group at nucleotide level as well as at the amino acid level (Figure 4.5). There were 34 positions within the nucleotide sequences of this region of *mutS* at which there was variation between the resistant isolates. Of these, there were 24 positions at which there was nucleotide variation only in gen.sp. 13TU isolate E51. There were a further three positions at which there was nucleotide variation only in *A.baumannii* isolate 790 (nuc 492, 513, 515), and a further position at which there was nucleotide variation only in *A. baumannii* isolate U7 (nuc 195).

Table 4.1 Details of the amino acid and nucleotide variation in the *mutS* gene of resistant clinical isolates compared to ADP1

Position	ADP1		Resistant isolates		Exceptions	
wrt ADP1	AA	Codon	AA	Codon	Isolate	Codon
67	Glu	GAG	Asn	AAC	-	-
78	Ala	GCT	Ser	TCA	-	-
91	Gln	CAA	Arg	CGA	E51	CGC
102	Gly	GGC	-	-	-	-
103	Glu	GAG	-	-	-	-
104	Asn	AAC	-	-	-	-
105	Ala	GCA	-	-	-	-
106	Gly	GGC	-	-	-	-
107	Ser	TCT	Val	GTT	E51	GTC
108	Arg	CGC	Thr	ACT	E51	ACA
109	Cys	TGT	Gly	GGC	-	-
111	Ala	GCC	Gly	GGC	E51	GGT
113	Met	ATG	Val	GTT	-	-
121	Ile	ATT	Leu	CTT	-	-
126	Ile	ATT	Leu	TTA	-	-
133	Gly	GGC	Thr	ACA	-	-
146	Gln	CAG	His	CAT	-	-
149	Lys	AAA	Gln	CAG	-	-
152	Ile	GGG	Phe	GGT	-	-
160	Ser	AGT	Gly	GGT	-	-
167	His	CAT	Gln	CAA	-	-
168	Glu	GAG	Asp	GAC	-	-
169	Phe	TTT	Tyr	TAC	-	-
171	Thr	ACT	Phe	CCG	790	CCC
175	Tyr	TAC	Phe	CCA	-	-

None of these observed nucleotide variations resulted in amino acid variation within the clinical isolates, although some (marked in bold in Figure 4.5) did occur in the codons where the R-type amino acid sequence varied compared to ADP1 (detailed in Table 4.1).

Figure 4.5 Nucleotide sequence alignment of the *mutS* of the resistant and multi-resistant clinical isolates

Numbering wrt ADP1. Nucleotide variations highlighted.
* indicates positions at which there is nucleotide variation between the resistant isolates.
Red and blue = conserved nuc variation. Green = non-conserved nuc variation.
BOLD = nucleotide changes occurring at positions where the R-type aa sequence varied compared to ADP1.

```
     181                                                                240
       *           *          *            *  *
779  CGAGGTAAAG CAAATGGCAA CCCGATTCCA ATGGCAGGTG TTCCCTACCA TTCAGCCGAA
783  CGGGGTAAAG CAAATGGCAA CCCGATTCCA ATGGCAGGTG TTCCCTACCA TTCAGCCGAA
789  CGGGGTAAAG CAAATGGCAA CCCGATTCCA ATGGCAGGTG TTCCCTACCA TTCAGCCGAA
790  CGGNNTAAAG CAAATGGCAA CC-GATTCCA ANGGCAGGTG TTCCCTACCA TTCAGCCGAA
868  CGGGGTAAAG CAAATGGCAA CCCGATTCCA ATGGCAGGTG TTCCCTACCA TTCAGCCGAA
U7   CGGGGTAAAG CAAACGGNAA CCCGATTCCA ATGGCAGGTG TTCCCTACCA TTCAGCCGAA
U45  CGAGGTAAAG CAAATGGCAA CCCGATTCCA ATGGCAGGTG TTCCCTACCA TTCAGCCGAA
E51  CGTGGTAAAG CAAATGGCAA CCCAATTCCA ATGGCGGGCG TTCCCTACCA TTCAGCCGAA
     241                                                                300
           *     *     *          *  *   *                    *     *  *
779  GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGC
783  GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGT
789  GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGC
790  GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGT
868  GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGC
U7   GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGT
U45  GGTTATTTGG CGCGTTTAGT AAAAGCCGGC CGAACTGTAG CTATTTGTGA GCAAGTCGGC
E51  GGTTATCTGG CACGTTTGGT AAAAGCAGGT CGCACTGTAG CTATTTGTGA ACAAGTGGGT
     301                                                                360
                              *  *         *  *       *  *
779  GAA------- --------GT TACTGGCAAA GGCCCGGTTG AGCGCAAAGT TGTTCGTATT
783  GAA------- --------GT TACTGGCAAA GGCCCGGTTG AACGCAAAGT TGTTCGTATT
789  GAA------- --------GT TACTGGCAAA GGCCCGGTTG AACGCAAAGT TGTTCGTATT
790  NAA------- --------GT TACTGGCAAA GGCCCGGTTG AACGCAAAGT TGTTCGTATT
868  GAA------- --------GT TACTGGCAAA GGCCCGGTTG AACGCAAAGT TGTTCGTATT
U7   GAA------- --------GT TACTGGCAAA GGCCCGGTTG AACGCAAAGT TGTTCGTATT
U45  GAA------- --------GT TACTGGCAAA GGCCCGGTTG AGCGCAAAGT TGTTCGTATT
E51  GAA------- --------GT CACAGGCAAA GGTCCTGTTG AGCGTAAAGT TGTTCGTATT
     361                                                                420
         *  *      *       *     *  *                     *   *
779  CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAATC GTCTAACCTT
783  CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAATC GTCTAACCTT
789  CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAGTC GTCTAACCTT
790  CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAATC GTCTAACCTT
868  CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAGTC GTCTAACCTT
U7   CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAGTC CTCTAACCTT
U45  CTTACACCGG GTACTTTAAC CGACGACGCA CTACTTACAA GTTATCAATC GTCTAACCTT
E51  CTTACCCCTG GTACATTAAC TGACGATGCC CTACTCACAA GTTATCAGTC TTCTAACCTT
     421                                                                480
       *
779  GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
783  GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
789  GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
790  GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
868  GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
U7   GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
U45  GTTGCGCTAT GTATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
E51  GTGGCGCTAT GCATCCATCA AAACCAGATC GGTTTTGCTT TACTCGACTT AAGTGCGGGT
     481                                                                540
               *                          * *             *         *
779  ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATCGA ACTGGCTCGC
783  ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATCGA ACTGGCTCGC
789  ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATTGA ACTGGCTCGC
790  ATTTTTAAAG TACAACAACA AGACTACAAA CCCGGACAAC TTCCAATCGA ACTCACCCGC
868  ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATTGA ACTGGCTCGC
U7   ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATTGA ACTCACCCGC
U45  ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATCGA ACTGGCTCGC
E51  ATTTTTAAAG TTCAACAACA AGACTACAAA CCGGAACAAC TTCCAATTGA ACTGGCACGC
```

4.2.4 Sequence Analysis – Intermediate isolates

Compared to the R-type *mutS* sequence, the amino acid sequences of the intermediate clinical isolates (comprising *A. baumannii*, gen.sp. 13TU and gen.sp. 3) also appeared highly conserved (Figure 4.6). There were 4 positions at which there was an amino acid variation compared to the R-type sequence, and all of these variations were in gen.sp. 3 isolate U80.

Figure 4.6 Alignment of the amino acid sequences of the *mutS* of intermediate clinical isolates compared to the sequence of the resistant clinical isolates

Numbering w.r.t. ADP1.
Variations compared to R-type highlighted.
* indicates position at which there is an amino acid variation compared to the R-type sequence.

```
          61                                                          120
              * *
R-type    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E13 (I)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E14 (I)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E41 (I)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U43 (I)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U51 (I)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U71 (I)   RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
U80 (I)   RGKASGRPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI

          121                                                         180
                      *          *
R-type    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E13 (I)   LTPGTLTEDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E14 (I)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E41 (I)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U43 (I)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U51 (I)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U71 (I)   LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
U80 (I)   LTPGTLTDDA LLSSYQSSNL VSLCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
```

Whilst there was a high level of conservation within the amino acid *mutS* sequences of the intermediate isolates, there was increased variation at the nucleotide level within these isolates, compared to that observed within the resistant isolates, as shown in Figure 4.7.

Figure 4.7 Alignment of the nucleotide sequences of the *mutS* of intermediate clinical isolates

Numbering wrt ADP1. Nucleotide changes highlighted.
* indicates positions at which there is nucleotide variation between the intermediate isolates.
Red, blue and Pink = conserved nuc variation. Green = non-conserved nuc variation.

```
     181                                                                240
       *  *     *  *    ***   *           *  *         *  *   *  *
E13  CGGGGTAAAGCA AATGGCAACC CGATTCCAAT GGCAGGTGTT CCCTACCATT CAGCCGAA
E14  CGTGGTAAAGCA AATGGCAACC CGATTCCAAT GGCAGGCGTT CCCTACCATT CAGCCGAA
E41  CGTGGTAAAGCA AATGGCAACC CAATTCCAAT GGCGGGCGTT CCCTACCATT CAGCCGAA
U43  CGTGGTAAAGCA AATGGCAACC CGATTCCAAT GGCAGGCGTT CCCTACCATT CAGCCGAA
U51  CGAGGTAAAGCA AATGGCAACC CGATTCCAAT GGCAGGTGTT CCCTACCATT CAGCCGAA
U71  CGGGGTAAAGCA AATGGCAACC CGATTCCAAT GGCAGGTGTT CCCTACCATT CAGCCGAA
U80  CGTGGCAAAGCC AGTGGCCGAC CTATTCCAAT GGCGGGTGTT CCCTATCACT CGGCAGAA
     241                                                                300
         * *  *   *  *  *      *  *   *     *  *       *  *       *  *
E13  GGTTATTTGGCG CGTTTAGTAA AAGCCGGCCG AACTGTAGCT ATTTGTGAGC AAGTCGGC
E14  GGTTATTTGGCG CGTTTAGTAA AAGCAGGCCG CACTGTAGCC ATTTGTGAAC AAGTGGGT
E41  GGTTATCTGGCA CGTTTGGTAA AAGCAGGTCG CACTGTAGCT ATTTGTGAAC AAGTGGGT
U43  GGTTATTTGGCG CGTTTAGTAA AAGCAGGCCG CACTGTAGCC ATTTGTGAAC AAGTGGGT
U51  GGTTATTTGGCG CGTTTAGTAA AAGCCGGCCG AACTGTAGCT ATTTGTGAGC AAGTCGGC
U71  GGTTATTTGGCG CGTTTAGTAA AAGCCGGCCG AACTGTAGCT ATTTGTGAGC AAGTCGGT
U80  GGTTATCTTGCT CGCCTTGTTA AAGCTGGCCG TACTGTCGCC ATTTGCGAAC AAGTTGGA
     301                                                                360
                           *   *  *      *  *     *   *
E13  GAA--------- ------GTTA CTGGCAAAGG CCCGGTTGAA CGCAAAGTTG TTCGTATT
E14  GAA--------- ------GTCA CCGGCAAAGG TCCTGTTGAG CGTAAAGTTG TTCGTATT
E41  GAA--------- ------GTCA CAGGCAAAGG TCCTGTTGAG CGTAAAGTTG TTCGTATT
U43  GAA--------- ------GTCA CCGGCAAAGG TCCTGTTGAG CGTAAAGTTG TTCGTATT
U51  GAA--------- ------GTTA CTGGCAAAGG CCCGGTTGAA CGCAAAGTTG TTCGTATT
U71  GAA--------- ------GTTA CTGGCAAAGG CCCGGTTGAA CGCAAAGTTG TTCGTATT
U80  GAA--------- ------GTGA CAGGTAAAGG CCCTGTTGAA CGTAAAGTTG TTCGTATT
     361                                                                420
       *  *  *      *     *   *  *  **     * **         *  *      *  *
E13  CTTACACCGGGT ACTTTAACCG AAGATGCACT ACTTACAAGT TATCAATCGT CTAACCTT
E14  CTTACCCCTGGT ACATTAACCG ACGATGCATT ACTCACAAGT TATCAGTCCT CTAATCTT
E41  CTTACCCCTGGT ACATTAACTG ACGATGCCCT ACTCACAAGT TATCAGTCTT CTAACCTT
U43  CTTACCCCTGGT ACATTAACCG ACGATGCATT ACTCACAAGT TATCAGTCCT CTAATCTT
U51  CTTACACCGGGT ACTTTAACCG ACGACGCACT ACTTACAAGT TATCAGTCGT CTAACCTT
U71  CTTACACCGGGT ACTTTAACTG ACGACGCACT ACTTACAAGT TATCAGTCCT CTAACCTT
U80  CTGACCCCTGGT ACATTAACTG ATGACGCATT ACTCAGCAGT TATCAATCAT CTAATCTC
     421                                                                480
       ** ** *  *   *              *                          *      *  *
E13  GTTGCGCTATGT ATCCATCAAA ACCAGATCGG TTTTGCTTTA CTCGACTT AAGTGCGGGT
E14  GTTGCGTTATGT ATCCATCAAA ACCAGATCGG TTTTGCTTTA CTCGACTT AAGTGCAGGT
E41  GTGGCGCTATGC ATCCATCAAA ACCAGATCGG TTTTGCTTTA CTCGACTT AAGTGCGGGT
U43  GTTGCGTTATGT ATCCATCAAA ACCAGATCGG TTTTGCTTTA CTCGACTT AAGTGCAGGT
U51  GTTGCGCTATGT ATCCATCAAA ACCAGATCGG TTTTGCTTTA CTCGACTT AAGTGCGGGT
U71  GTTGCGCTATGT ATCCATCAAA ACCAGATCGG TTTTGCTTTA CTCGACTT AAGTGCGGGT
U80  GTTTCATTGTGC ATTCATCAAA ACCAGATTGG TTTTGCTTTA CTCGACTT GAGTGCAGGC
     481                                                                540
                              *     *   *  *   * *   *  *       *  *  *
E13  ATTTTTAAAGTT CAACAACAAG ACTACAAACC GGAACAACTT CCAATCGAAC TGGCTCGC
E14  ATTTTTAAAGTT CAACAACAAG ACTACAAACC GGAACAACTT CCAATTGAAC TGGCACGC
E41  ATTTTTAAAGTT CAACAACAAG ACTACAAACC GGAACAACTT CCAATTGAAC TGGCACGC
U43  ATTTTTAAAGTT CAACAACAAG ACTACAAACC GGAACAACTT CCAATTGAAC TGGCACGC
U51  ATTTTTAAAGTT CAACAACAAG ACTACAAACC GGAACAACTT CCAATTGAAC TGGCTCGC
U71  ATTTTTAAAGTT CAACAACAAG ACTACAAACC GGAACAACTT CCAATTGAAC TGGCTCGC
U80  ATTTTTAAAGTT CAACAACAAG ATTACAAGCC AGAGCAATTG CCTATTGAAC TTGCGCGT
```

Within the examined region, there were 74 positions at which one or more of the intermediate clinical isolates had a nucleotide variation within their *mutS* sequence compared to the consensus nucleotide sequence. Of these there were 38 positions at which there was a nucleotide variation only in gen.sp. 3 isolate U80, the same isolate which contained the only amino acid variations observed within the intermediate isolates.

4.2.5 Sequence Analysis – Sensitive isolates

In comparison to the resistant and intermediate isolates the sensitive isolates (comprising *A. baumannii*, gen.sp. 3, *A. johnsonii* and *A. junii*) were much more varied compared to each other at both the nucleotide and amino acid level. Compared to the R-type amino acid sequence observed in the *mutS* of the resistant and most of the intermediate isolates, there were 27 positions at which one or more of the sensitive isolates had a different amino acid. This is shown in Figure 4.8.

Of these 27 positions there were none at which all sensitive isolates had the same amino acid variation compared to the R-type sequence. There were also nine instances where there was a non-conserved amino acid variation observed in only one of the sensitive isolates.

Figure 4.8 Alignment of the amino acid sequences of the *mutS* of sensitive clinical isolates compared to the sequence of the resistant clinical isolates

Numbering w.r.t. ADP1. Variations highlighted.
* indicates position at which there is amino acid variation compared to the R-type sequence.
Red = conserved variations in the sensitive isolates compared to the R-type sequence.
Blue = conserved variations in the sensitive isolates compared to the R-type sequence.
Green = non-conserved variations in the sensitive isolates compared to the R-type sequence.

```
          61                                                          120
             ** *           *           *  *  *    *                  *
R-type    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E9   (S)  RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E10  (S)  RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E15  (S)  RGKANGTPIP MAGVPYHSAE GYLARLVKAG ETVAICEQVG E-----VTGK GPVDRQVVRI
E21  (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
E26  (S)  RGKTNGEPIP MAGVPYHSAE GYLARLVKSG RTVAICEQVG E-----VTGK GPVERKVVRV
E33  (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
U66  (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI

          121                                                         180
          *            *** *       ** * *  *     *   *     ** * **  *
R-type    LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E9   (S)  LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E10  (S)  LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E15  (S)  LTPGTLTD-A LLSSHQSSNL VALCFQQNQV GIALLDLGAG IFKVQQQDYK PEQLTIELAR
E21  (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
E26  (S)  LTPGTLTDDA LLSSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQTYK PEQLPIELAR
E33  (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
U66  (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
```

However, despite the variation within the sensitive population conserved *mutS* sequences with the same amino acid variations compared to the R-type sequence were observed in certain groups of isolates, as depicted in Figure 4.9. There were two groups with conserved *mutS* amino acid sequences within the sensitive isolates comprising: E9 and E10 (both identified in Chapter 3 as probable gen.sp. 3); and E21, E33 and U66 (identified in Chapter 3 as *A. johnsonnii*, *A. johnsonnii* and *A. baumannii* respectively). E15 (identified as *A. radioresistens*) and E26 (identified as *A. junii*) had similarities to both sequence types, but did not fit in to either group.

Figure 4.9 Conserved *mutS* sequences within the sensitive amino acid isolates

```
          61                                                        120
E9   (S)  RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E10  (S)  RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI

          121                                                       180
E9   (S)  LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E10  (S)  LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR



          61                                                        120
E21  (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
E33  (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
U66  (S)  RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI

          121                                                       180
E21  (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
E33  (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
U66  (S)  ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
```

Conserved changes compared to the R-type sequence are highlighted.

Interestingly, of the 27 positions at which there was amino acid variation compared to the R-type sequence, there were 17 at which the amino acid was the same as one or more of the non-clinical isolates. This is marked in Figure 4.10 which shows the alignment of the amino acid *mutS* sequences of the sensitive isolates compared to the non-clinical strains. All of the variations found within the sensitive isolates and where they corresponded to the non-clinical isolates are detailed in Table 4.2.

Figure 4.10 Alignment of the amino acid *mutS* sequences of the sensitive clinical isolates compared with the resistant isolates' and non-clinical sequences

Numbering w.r.t. ADP1. Variations highlighted.
+ indicates positions at which there is amino acid variation in one or more of the sensitive isolates compared to the R-type sequence, where this difference corresponds to the sequence of one or more of the non-clinical strains.
Blue = highlighting where non-clinical isolates match sensitive clinical isolates at positions where they differ to the R-type sequence.

```
            61                                                        120
                  +           +           +  +  +    +                 +
ADP1     (C) RGKANGEPIP MAGVPYHAAE GYLARLVRAG QTVAICEQVG EGENAGSRCK APMERKVVRI
93A2     (C) RGKANGEPIP MAGVPYHAAE GYLARLVRAG QTVAICEQVG EGENAGSRGK APMERKVVRI
AD321    (C) RGKANGEPIP MAGVPYHAAE GYLARLVKAG QTVAICEQVG EGESAGSRGK APMERKVVRI
AC423D(C) RGKANGEPIP MAGVPYHAAE GYLARLVKKG ETVVICEQIG E-----VTGK APVERGVVRI
R-type      RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E9       (S) RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E10      (S) RGKASGQPIP MAGVPYHAAE GYLARLVKAG RTVAICEQVG E-----VTGK GPVERKVVRI
E15      (S) RGKANGTPIP MAGVPYHSAE GYLARLVKAG ETVAICEQVG E-----VTGK GPVDRQVVRI
E21      (S) RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
E26      (S) RGKTNGEPIP MAGVPYHSAE GYLARLVKSG RTVAICEQVG E-----VTGK GPVERKVVRV
E33      (S) RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI
U66      (S) RGKANGQPIP MAGVPFHAAE GYLARLVKKG ETVVICEQIG E-----VTGK GPVERGVVRI

            121                                                       180
            +           +++         ++      +         +          +   +
ADP1     (C) ITPGTITDDA LLGSYQSSNL VALCIQQNKI GIALLDLSAS IFKVQQHEFK TEQLYIELAR
93A2     (C) ITPGTITDDA LLGSYQSSNL VALCIQQNKI GIALLDLSAS IFKVQQHEFK TEQLYIELAR
AD321    (C) ITPGTITDDA LLGSYQSSNL VALCIQQNKI GLALLDLSAS IFKVQEHDFK TEQLAIELSR
AC423D(C) ITPGTLTDDA LLTAHQSSNL VALCVQQNEI GIALLDLSAG LFKVQQQEFQ LEQLGIELSR
R-type      LTPGTLTDDA LLTSYQSSNL VALCIHQNQI GFALLDLSAG IFKVQQQDYK PEQLPIELAR
E9       (S) LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E10      (S) LTPGTLTDDA LLGSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQDYK PEQLAIELAR
E15      (S) LTPGTLTD-A LLSSHQSSNL VALCFQQNQV GIALLDLGAG IFKVQQQDYK PEQLTIELAR
E21      (S) ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
E26      (S) LTPGTLTDDA LLSSYQSSNL VALCIQQNQI GFALLDLSAG IFKVQQQTYK PEQLPIELAR
E33      (S) ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
U66      (S) ITPGTLTDDA LLGAHQTSNL VALCVHQQQI GIALLDLSAG LFKVQQIDYD LSQLAIELAR
```

Table 4.2 Details of the variation in the *mutS* sequences of the sensitive isolates compared to the *mutS* sequences of the resistant isolates' and non-clinical strains

Position (wrt ADP1)	R-type		Changes of sensitive isolates compared to R-type			ADP1		If different to R, same as non-clinical?	
	AA	Codon	Isolate	AA	Codon	AA	Codon	Y/N	Non-clin AA
64	Ala	GCA	E26	Thr	ACA	Ala	GCT	N	-
65	Asn	AAT	E9	Ser	AGT	Asn	AAT	N	-
			E10	Ser	AGT			N	-
67	Asn	AAC	E9	Gln	CAG	Glu	GAG	N	-
			E10	Gln	CAG			N	-
			E21	Gln	CAG			N	-
			U66	Gln	CAG			N	-
			E33	Gln	CAG			N	-
			E26	Glu	GAA			Y	Glu
			E15	Thr	ACG			N	-
78	Ser	TCA	E9	Ala	GCA	Ala	GCT	Y	Ala
			E10	Ala	GCA			Y	
			E21	Ala	GCT			Y	
			U66	Ala	GCT			Y	
			E33	Ala	GCT			Y	
			E15	Ala	TCT			Y	
89	Ala	GCC	E26	Ser	TCA	Ala	GCA	N	-
			E21	Lys	AAA			Y	Lys
			U66	Lys	AAA			Y	
			E33	Lys	AAA			Y	
91	Arg	CGA	E21	Glu	CGA	Gln	CAA	Y	Glu
			U66	Glu	GAA			Y	
			E33	Glu	GAA			Y	
			E15	Glu	GAG			Y	
94	Ala	GCT	E21	Val	GTG	Ala	GCT	Y	Val
			U66	Val	GTG			Y	
			E33	Val	GTG			Y	
99	Val	GTC	E21	Ile	ATC	Val	GTA	Y	Ile
			U66	Ile	ATC			Y	
			E33	Ile	ATC			Y	
116	Lys	AAA	E21	Gly	GGT	Lys	AAA	Y	Gly
			U66	Gly	GGT			Y	
			E31	Gly	GGT			Y	
			E15	Gln	CAA			N	-
121	Leu	CTT	E21	Ile	ATT	Ile	ATT	Y	Ile
			U66	Ile	ATT			Y	
			E33	Ile	ATT			Y	
133	Thr	ACA	E9	Gly	GGA	Gly	GGC	Y	Gly
			E10	Gly	GGA			Y	
			E21	Gly	GGT			Y	
			U66	Gly	GGT			Y	
			E33	Gly	GGT			Y	
			E26	Ser	TCA			N	-
			E15	Ser	AGC			N	-
134	Ser	AGT	E21	Ala	GCT	Ser	AGT	Y	Ala
			U66	Ala	GCT			Y	
			E33	Ala	GCT			Y	

Table 4.2 continued

Position (wrt ADP1)	R-type		Changes of sensitive isolates compared to R-type			ADP1		If different to R, same as non-clinical?	
	AA	Codon	Isolate	AA	Codon	AA	Codon	Y/N	Non-clin AA
135	Tyr	TAT	E21	His	CAT	Tyr	TAT	Y	His
			U66	His	CAT			Y	
			E33	His	CAT			Y	
			E15	His	CAT			Y	
137	Ser	TCC	E21	Thr	ACC	Ser	TCT	N	-
			U66	Thr	ACC			N	-
			E31	Thr	ACC			N	-
145	Ile	ATC	E21	Val	GTT	Ile	ATT	Y	Val
			U66	Val	GTT			Y	
			E33	Val	GTT			Y	
			E15	Phe	TTC			N	-
146	His	CAT	E9	Gln	CAA	Gln	CAG	Y	Gln
			E10	Gln	CAA			Y	
			E26	Gln	CAA			Y	
			E15	Gln	CAG			Y	
148	Asn	AAC	E21	Gln	CAA	Asn	AAT	N	-
			E33	Gln	CAA			N	-
			U66	Gln	CAA			N	-
150	Ile	ATC	E15	Val	GTC	Ile	ATT	N	-
152	Phe	TTT	E21	Ile	ATT	Ile	ATT	Y	Ile
			U66	Ile	ATT			Y	
			E33	Ile	ATT			Y	
			E15	Ile	ATT			Y	
158	Ser	AGT	E15	Gly	GGT	Ser	AGT	N	-
161	Ile	ATT	E21	Leu	TTA	Ile	ATA	Y	Ile
			U66	Leu	TTA			Y	
			E33	Leu	TTA			Y	
167	Gln	CAA	E21	Ile	ATC	His	CAT	N	-
			U66	Ile	ATC			N	-
			E33	Ile	ATC			N	-
168	Asp	GAC	E26	Thr	ACA	Glu	GAG	N	-
170	Lys	AAA	E21	Asp	GAC	Lys	AAA	N	-
			U66	Asp	GAC			N	-
			E33	Asp	GAC			N	-
171	Pro	CCG	E21	Leu	TTA	Thr	ACT	Y	Leu
			U66	Leu	TTA			Y	
			E33	Leu	TTA			Y	
172	Glu	GAA	E21	Ser	AGT	Glu	GAA	N	-
			U66	Ser	AGT			N	-
			E33	Ser	AGT			N	-
175	Pro	CCA	E9	Ala	GCC	Tyr	TAC	Y	Ala
			E10	Ala	GCC			Y	
			E21	Ala	GCC			Y	
			U66	Ala	GCC			Y	
			E33	Ala	GCC			Y	

As shown in Table 4.2, within the sensitive isolates there are 60 instances where an isolate varied compared to the resistant isolates' *mutS* sequence, but corresponded instead to one or more of the non-clinical isolates.

The sensitive isolates were also markedly more varied than the other clinical isolates at the nucleotide level, as shown in Figure 4.11. There were 146 positions at which there was nucleotide variation between the isolates. At 40 of these positions, there were non-conserved changes, where the nucleotide did not match any other of the sensitive clinical isolates.

Figure 4.11 Alignment of the nucleotide sequences of the *mutS* of sensitive clinical isolates

Numbering w.r.t. ADP1. * indicates position at which there is aa variation compared to the R-type sequence.
Red, Blue and Pink = conserved variations within the sensitive isolates compared to the majority.
Green = non-conserved variations in the sensitive isolates compared to the majority.

```
      181                                                                240
        *   *    *   * **  *** *  *     *       *  *   *  * **    * *  *  *
E9    CGTGGCAAAG CCAGTGGTCA GCCAATTCCA ATGGCGGGTG TACCTTATCA TGCAGCAGAG
E10   CGCGGCAAAG CCAGTGGTCA GCCAATTCCA ATGGCAGGTG TACCTTATCA TGCAGCAGAG
E15   CGTGGTAAAG CCAACGGTAC GCCTATTCCT ATGGCGGGGG TGCCCTATCA TTCTGCCGAA
E21   CGCGGTAAAG CCAATGGCCA GCCAATTCCG ATGGCCGGTG TACCTTTCCA TGCTGCTGAA
E26   CGTGGCAAAA CAAATGGTGA ACCAATTCCA ATGGCAGGTG TTCCCTACCA TTCCGCTGAA
E33   CGNGGTAAAG CCAATGGCCA GCCAATTCCG ATGGCCGGTG TACCTTTCCA TGCTGCTGAA
U66   CGCGGTAAAG CCAATGGCCA GCCAATTCCG ATGGCCGGTG TACCTTTCCA TGCTGCTGAA
      241                                                                300
        *   * *   *  ** *   *  **** *  *** *  *  **  *  *   *  ** *
E9    GGCTATCTGG CTCGTTTAGT CAAAGCTGGC CGTACTGTCG CCATCTGCGA ACAAGTTGGT
E10   GGCTATCTCG CTCGTTTAGT CAAAGCTGGC CGTACTGTCG CCATCTGCGA ACAAGTTGGT
E15   GGGTATCTGG CCCGGCTGGT AAAAGCCGGT GAGACCGTGG CCATCTGTGA ACAGGTGGGT
E21   GGCTATTTGG CTCGTCTGGT GAAAAAAGGC GAAACGGTGG TGATCTGCGA GCAGATCGGT
E26   GGATATTTAG CACGTTTAGT AAAGTCAGGT CGTACCGTTG CAATTTGTGA ACAAGTTGGT
E33   GGCTATCTGG CTCGTCTGGT GAAAAAAGGC GAAACGGTGG TGATCTGCGA GCAGATCGGT
U66   GGCTATCTGG CTCGTCTGGT AAAAAAAGGC GAAACGGTGG TGATCTGCGA GCAGATCGGT
      301                                                                360
        *                   *  *  *     *  *      *   ***   *  *  ** *
E9    GAG------- --------GT CACAGGCAAA GGTCCAGTTG AGCGTAAAGT AGTCCGTATC
E10   GAG------- --------GT CACAGGCAAA GGTCCGGTTG AGCGTAAAGT AGTACGTATC
E15   GAA------- --------GT GACCGGTAAA GGTCCGGTTG ACCGTCAAGT CGTGCGTATC
E21   GAA------- --------GT GACTGGTAAA GGCCCAGTTG AACGTGGTGT CGTCCGAATC
E26   GAA------- --------GT TACAGGTAAA GGACCTGTCG AGCGTAAAGT AGTCCGAGTT
E33   GAA------- --------GT GACTGGTAAA GGCCCAGTTG AACGTGGTGT CGTCCGAATC
U66   GAA------- --------GT GACTGGTAAA GGCCCAGTTG AACGTGGTGT CGTCCGAATC
      361                                                                420
      * *  *  *   *  *      *  *     * * ** ** ** *** *  *** *  *  ** *
E9    TTAACGCCAG GCACATTAAC CGATGATGCT TTATTGGGAA GTTACCAATC GTCGAATCTG
E10   TTAACGCCAG GCACATTAAC CGATGATGCT TTATTGGGAA GTTACCAATC GTCGAATCTG
E15   CTCACCCCCG GTACCTTAAC CGATNATGCT CTGCTCAGCT CACATCAGAG CTCCAATCTG
E21   ATTACCCCTG GAACCTTAAC CGATGATGCC TTATTAGGTG CTCATCAAAC CTCCAACCTG
E26   TTGACACCTG GTACCTTAAC TGACGATGCG CTTTTATTCAA GTTATCAATC CTCAAATTTA
E33   ATTACTCCTG GAACCTTAAC CGATGATGCC TTATTAGGTG CTCATCAAAC CTCCAACCTG
U66   ATTACCCCTG GAACCTTAAC CGATGATGCC TTATTAGGTG CTCATCAAAC CTCCAACCTG
      421                                                                480
          ** *   ** *  *   ** *  ** *   ** *  **  *  *   *  **    *  *
E9    GTTGCGCTCT GTATTCAACA AAATCAAATT GGTTTCGCCT TACTCGACTT GAGTGCAGGC
E10   GTTGCGCTCT GTATTCAACA AAATCAAATT GGTTTCGCCT TACTCGACTT GAGTGCAGGA
E15   GTTGCGTTAT GCTTCCAGCA AAATCAGGTC GGTATTGCAC TGCTGGACTT AGGTGCAGGT
E21   GTTGCTCTGT GTGTTCACCA GCAACAAATC GGAATTGCAC TTCTTGACCT GAGTGCTGGT
E26   GTTGCTCTGT GTATACAACA AAATCAAATT GGTTTTGCAT TACTTGACTT GAGTGCAGGT
E33   GTTGCTCTGT GTGTTCACCA GCAACAAATC GGAATTGCAC TTCTTGACCT GAGTGCCGGT
U66   GTTGCTCTGT GTGTTCACCA GCAACAAATC GGAATTGCAC TTCTTGACCT GAGTGCCGGT
      481                                                                540
      * *  *       *  *  *** **** *  * * ****** *   ** *  *   ** *  *  *
E9    ATCTTTAAAG TTCAACAACA AGACTATAAA CCTGAACAAT TGGCCATTGA ACTGGCGCGT
E10   ATCTTTAAAG TTCAACAACA AGACTATAAG CCTGAACAAT TGGCCATTGA ACTGGCACGT
E15   ATTTTTAAAG TTCAGCAGCA GGACTATAAA CCGGAACAGT TAACGATTGA GCTGGCGCGA
E21   TTATTCAAAG TCCAGCAAAT CGATTATGAC TTAAGTCAGT TAGCCATTGA GTTGGCACGC
E26   ATTTTTAAAG TTCAACAACA GACATATAAA CCAGAGCAAT TGCCAATAGA ACTTGCTCGC
E33   TTATTTAAAG TCCAGCAAAT CGATTATGAC TTAAGTCAGT TAGCCATTGA GTTGGCACGC
U66   TTATTTAAAG TCCAGCAAAT CGATTATGAC TTAAGTCAGT TAGCCATTGA GTTGGCACGC
```

4.2.6 *mutS* Sequence of Outbreak Strains

As mentioned (Section 2.1) several representatives of *A. baumannii* outbreak strains were gifted by Dr Jane Turton and their sensitivities determined (Section 3.2.1). Part of the *mutS* genes of these strains were also sequenced and examined, and compared to the R-type amino acid sequence, as shown in Figure 4.12.

Figure 4.12 Alignment of the *mutS* gene of several *A. baumannii* outbreak strains compared to the R-type sequence of clinical isolates examined above

```
          61                                                          120
R-type    RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL
JTA       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL
JTB       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL
JTC       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL
JT3       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL
JT4       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL
JT6       RGKANGNPIP MAGVPYHSAE GYLARLVKAG RTVAICEQVG EVTGKGPVER KVVRILTPGTL

          121                                                         180
R-type    TDDALLTSYQ SSNLVALCIH QNQIGFALLD LSAGIFKVQQ QDYKPEQLPI ELARLMPSEIL
JTA       TDDALLTSYQ SSNLVALCIH QNQIGFAL
JTB       TDDALLTSYQ SSNLVALCIH QNQIGFALLD L
JTC       TDDALLTSYQ SSNLVALCIH QNQIGFALLD LSAGIFKVQQ QDYKPEQLPI E
JT3       TDDALLTSYQ SSNLVALCIH QNQIGFALLD LSAGIFKVQQ QDYKPEQLP
JT4       TDDALLTSYQ SSNLVALCIH QNQIGFALLD L
JT6       TDDALLTSYQ SSNLVALCIH QNQIGFALLD
```

As is apparent, the *mutS* amino acid sequence of the examined section of the outbreak strains is identical to that of the R-type sequence found in the resistant, multi-resistant and most intermediate clinical isolates.

4.2.7 Sequences Analysis Summary

Specific positions of variation were evident when the amino acid sequences of the *mutS* gene of the clinical isolates were compared to ADP1. As shown in Figure 4.2 there were 39 positions at which there was amino acid variation of one or more of the clinical isolates compared to ADP1. At 10 of these positions all clinical isolates had a variation, at 12 the majority had a conserved variation, and at 16 there was variation compared to ADP1 but the amino acid change corresponded to one or more of the other non-clinical isolates.

Variation in the *mutS* gene was also observed within the clinical isolates themselves, with 87 instances at 23 positions where one or more isolates varied in comparison to the consensus sequence (Figure 4.3). The majority of the variations occurred in the sensitive isolates, with only two occurring in the intermediate isolate U80.

Looking at the differences in terms of sensitivities, the resistant and multi-resistant isolates had highly conserved *mutS* sequences at both amino acid and nucleotide level (Figures 4.4, 4.5 and Table 4.1) with no amino acid differences and 34 positions of nucleotide variation, the majority of which (24 of 34) occurred only in isolate E51.

The majority of the intermediate isolates were also highly conserved, with the same amino acid sequence as that of the resistant isolates, with the exception of U80 which had 4 amino acid variations (Figure 4.6). There was, however, more variation at the

nucleotide level within the intermediate isolates, with 74 positions at which there was nucleotide variation compared to the consensus sequence (Figure 4.7).

The sensitive isolates were much more varied in both amino acid and nucleotide *mutS* sequences, with 27 positions of amino acid variation compared to the R-type sequence and 146 positions at which there was nucleotide variation between the sensitive isolates. Additionally, at 17 of the 27 positions mentioned above the amino acid variation correlated with one or more of the non-clinical strains. (Figures 4.8, 4.10 and Table 4.2).

Looking at the results in terms of species, whilst all of the clinical isolates differed from ADP1 and the other non-clinical isolates, *A. baumannii* and gen.sp. 13TU appeared to have a highly conserved amino acid *mutS* sequence (which has been referred to as the R-type sequence), whilst the other *Acinetobacter* species (the sensitive isolates and the intermediate isolate U80) had various amino acid differences compared to this R-type sequence. Additionally, of the nucleotide variation seen in the resistant isolates, 24 out of 34 variations occurred only in the gen.sp. 13TU isolate E51; likewise the only amino acid variation in the intermediate isolates occurred in the gen.sp. 3 isolate U80. Importantly, the *A. baumannii* outbreak strains all had a *mutS* amino acid sequence identical to the R-type sequence examined clinical isolates.

4.3 Discussion

The aims set out at the beginning of the chapter were: to examine the *mutS* gene of clinical *Acinetobacter* isolates; to determine whether there was variation both compared to non-clinical strains and within the clinical population; and to examine whether this variation correlated with different antibiotic susceptibilities.

It was relevant to examine the *mutS* gene of clinical *Acinetobacter* isolates of different species for this investigation, as discussed in Sections 1.3 and 4.1. Despite the predominance of *A. baumannii* in hospital infections other species, especially gen.sp. 13TU, are also frequently isolated from infections and further species also form part of the clinical population of *Acinetobacter*. The representative isolates used were genetically unrelated, as determined by PFGE of *Apa*I digested DNA, and comprised resistant, intermediate and sensitive susceptibilities as determined from the MICs of several tested antibiotics (Chapter 3).

From the results it was apparent that the *mutS* gene sequence of clinical isolates did vary compared to the non-clinical strains. All examined clinical isolates had in common a section of five deleted amino acids (aa 102-106 inclusive) compared to ADP1, also observed in one of the non-clinical strains (AC423D). Indels have previously been reported within ADP1 and other non-clinical isolates of *Acinetobacter*, and it was hypothesised that they set it apart from other Gram-negative bacteria (Section 1.5.5; Young & Ornston, 2001). However the clinical *Acinetobacter* isolates examined here did

not share the extra five amino acids (aa 102-106) suggesting that the difference is not as clear as supposed.

Variation was also apparent within the clinical isolates themselves. Whilst the comparison was predominantly with ADP1 the non-clinical isolates also varied in terms of their *mutS* gene and there were instances where an amino acid variation within the clinical isolates corresponded to one or more of the other non-clinical isolates. This was particularly evident in the sensitive *Acinetobacter* spp isolates when compared to the R-type sequence found in the resistant and intermediate isolates (which comprised *A.baumannii*, gen.sp. 13TU and gen.sp. 3); there were several positions at which the sensitive isolates varied compared to the other clinical isolates but instead the amino acid corresponded to one or more of the non-clinical isolates.

Looking at the *Acinetobacter* spp clinical isolates together, the similarity of the *mutS* sequences of some of the sensitive *Acinetobacter* spp isolates to the non-clinical isolates may be an indication that these isolates are transitional between the non-clinical species of *Acinetobacter* and the resistant isolates, in terms of their *mutS* sequence. In addition, most of the *A. baumannii* intermediate isolates had the same R-type amino acid sequence as the resistant and multi-resistant isolates; however, at the nucleotide level there was still greater variation seen in the intermediate compared to the resistant isolates. This supports the idea that, in terms of the *mutS* gene, they may be at an intermediate stage between the non-clinical and sensitive isolates and the highly conserved *mutS* sequence type associated with multi-resistant clinical isolates. The

intermediate isolates may therefore be an example of the next stage of progression between the *mutS* gene of sensitive isolates and that found in the resistant isolates.

In terms of species, the R-type *mutS* amino acid sequence was found only in *A.baumannii* and gen.sp. 13TU clinical isolates. These species are genetically highly related, both considered important in the hospital environment (though *A. baumannii* more so), and are both part of the Acb complex. Interestingly U80, an intermediate isolate with only 4 amino acid differences compared to the R-type sequence, is gen.sp. 3; also part of the Acb complex, gen.sp. 3 is increasingly considered potentially relevant in the clinical environment (Section 1.3; Tjernberg & Ursing, 1989; Gerner-Smidt *et al*, 1991; Dijkshoorn *et al*, 2007). The sensitive isolates E21 and E33, both identified as *A. johnsonnii*, had identical amino acid *mutS* sequences, though U66 (a sensitive *A. baumannii* isolate) also shared this sequence.

Hence, whilst it could be argued that the *mutS* differences may be species specific, an identical R-type sequence was not found in the sensitive U66 *A. baumannii* isolate (identified as such in Chapter 3 following a positive $bla_{\text{OXA-51-like}}$ PCR and 16S-23S intergenic rRNA sequence analysis). Additionally, whilst *A. baumannii* and gen.sp. 13TU are highly related, they are still considered separate species, and all of these isolates had an identical R-type *mutS* amino acid sequence. Furthermore, in addition to intermediate isolate U80, sensitive isolates E9 and E10 were also identified as probable gen.sp. 3. (Chapter 3). U80 was the only intermediate isolate to differ (at four aa positions) from the R-type *mutS* sequence, and one of these changes (aa 65) was in

common with that observed in E9 and E10 (see Figure 4.9), but the *mutS* sequences were not identical within isolates from these species. Therefore, whilst it could be argued that this difference is species-specific, taking the results above and species information from Chapter 3 together, this seems unlikely.

As discussed in Section 1.3, the taxonomy and speciation of *Acinetobacter* spp is historically complicated, and even with the advent of genotypic typing methods, there can still be uncertainty about speciation, with members of the Acb complex frequently grouped together. In terms of the *mutS* amino acid sequence found here, and corresponding sensitivities, the caution regarding this grouping of different species seems supported, as sensitive and intermediate isolates of gen.sp. 3 would thus be grouped with resistant and multi-resistant *A. baumannii* and 13TU isolates.

From these observations it appears, at least in this selection of the hospital population of *Acinetobacter* spp, that there is greater conservation of the *mutS* gene with higher levels of resistance; there was variation in the *mutS* gene within the clinical isolates but the resistant and multi-resistant isolates, and most importantly the outbreak strains, all had an identical R-type *mutS* amino acid sequence. Whilst the same was observed in most intermediate isolates too, they were more varied in terms of their nucleotides, supporting the idea of homogeneity with increased resistance. Given the reported clonal spread of multi-resistant *A. baumannii* isolates (Section 1.3.4; Coehlo *et al,* 2004), some of which were examined here, homogeneity is likely to be a characteristic of the most successful *Acinetobacter* isolates, hence the conserved R-type *mutS* sequence seems significant.

The correlation of the highly conserved *mutS* type with the most clinically relevant *Acinetobacter* isolates (that is, the R-type *mutS* sequence was found in outbreak *A. baumannii*, and other resistant and multi-resistant *A. baumannii* and gen.sp. 13TU clinical isolates) may be indicative of the relevance of *mutS* in the ability of these species, and *A. baumannii* in particular, to predominate in the clinical environment. As discussed in Section 1.3.5, whilst the individual antibiotic resistance mechanisms of *Acinetobacter* spp are well characterised, it is the remarkable ability of *A. baumannii* in particular to acquire resistance which is fundamental to its prevalence, and the reasons behind this are unclear; it may be that, similar to reports in other species (Section 1.5), defects in the *mutS* gene are associated with this ability.

Regarding the aims of this Chapter, these observations show that the *mutS* gene of clinical *Acinetobacter* spp does differ from that of non-clinical strains, and that there is also sensitivity-associated variation in the *mutS* gene of clinical isolates. A highly conserved, novel *mutS* amino acid sequence was found in outbreak, multi-resistant and resistant isolates. As such these results support the hypothesis that differences in the *mutS* gene may be a factor in the ability of sub-populations of *Acinetobacter* spp in the clinical environment to rapidly acquire resistance. Chapter 5 examines the mutation potential of these isolates to determine whether the observed *mutS* differences are correlated with differences in ability to develop resistance.

Chapter 5: *Acinetobacter* mutation studies

5.1 Introduction

In the previous chapter it was determined that there were differences in the *mutS* gene of clinical *Acinetobacter* spp isolates compared to the non-clinical strains and that there was sensitivity-associated variation of the *mutS* gene within the clinical isolates. This chapter examines whether the different *mutS* types are correlated with differences in the ability of the isolates to develop antibiotic resistance.

As discussed in Section 1.5.3, although the importance of mutation in antibiotic resistance development is clear, and hypermutation is also increasingly considered an important factor, increased mutation rates ought not to be considered in isolation as a direct cause of resistance development (Matic *et al*, 1997; Denamur *et al*, 2002; O'Neill & Chopra, 2002; Martinez & Baquero, 2000; Woodford & Ellington, 2007).

As such, mutation potential must also be measured in terms of ability to develop clinically significant resistance. Fluoroquinolones, as discussed in Section 1.4, are of particular interest regarding *Acinetobacter* spp resistance, with fluoroquinolone resistance highlighted as a possible risk factor for epidemic behaviour in *A. baumannii* (Wisplinghoff *et al*, 2003).

In *Acinetobacter*, the primary mechanism of resistance to fluoroquinolones is target site mutation in the *gyrA* gene, most commonly Serine-83 to Leucine (Vila *et al*, 1995). Mutations in *parC* are also implicated as a secondary target in fluoroquinolone resistance development but their role is less certain (see Section 1.4.5). Over-expression of efflux systems is also a mechanism of *Acinetobacter* resistance to fluoroquinolones, however such changes in expression are transient and hence not associated with lasting clinically significant resistance (Section 1.4.6).

In *gyrA*, the loss of the *Hinf*I restriction site GANTC at codons 82 and 83 when the expected target site mutation is present can be readily indicated by restriction analysis with *Hinf*I restriction enzyme. The possible existence of a target site mutation is suggested by unrestricted *gyrA* PCR products as seen on an agarose gel and confirmed by sequence analysis (Sections 1.4.5 and 2. 10.3).

Fluoroquinolone resistance development, as measured by MICs after challenge with ciprofloxacin and target site mutation in the *gyrA* gene (Section 2.10), is used as a tool here to examine the mutation potential of the representative *Acinetobacter* spp isolates detailed in Chapters 3 and 4.

5.2 Results

5.2.1 Generation of First Step Mutants

All sensitive and intermediate *Acinetobacter* spp isolates examined in Chapter 4 were challenged with 2 x MIC of ciprofloxacin to generate mutants, as described in Section 2.10. Randomly-picked mutants thus generated were stored and their ciprofloxacin MICs determined as described in Section 2.7. During the generation of first step mutants isolates were also challenged with excess ciprofloxacin; only U43 produced any mutants in response to this challenge. These were stored and labelled U43+, and are discussed in Section 5.2.9 below.

5.2.2 Mutation Frequencies

Spontaneous mutation frequencies were calculated as the ratio of potential mutants to viable colonies for each isolate (Section 2.10). Table 5.1 shows the mean mutation frequencies of each isolate, expressed as number of resistant mutants recovered as a fraction of total viable count.

It is clear that the mutation frequency varies within the *Acinetobacter* isolates examined, from 6×10^{-8} to 7.22×10^{-4}, indicating that there is potential that some isolates in comparable conditions could have much higher mutation frequencies. Compared to the non-clinical strain ADP1, all of the intermediate isolates with the R-type *mutS* sequence had higher mutation frequencies. Four of the sensitive isolates, without the R-type *mutS*

sequence, also had higher mutation frequencies than ADP1, with the highest observed mean mutation frequencies in sensitive isolates E10 and U66, followed by U71, E9 and E26.

Table 5.1 Mean mutation frequencies of parent strains

* indicates repeats

Isolate		Mean mutation frequency	Isolate		Mean mutation frequency
ATCC 19606	C	7.86×10^{-7}	E26	S	1.01×10^{-4}
ATCC 19606 *	C	3.30×10^{-5}	E33	S	6.00×10^{-8}
ADP1	C	3.45×10^{-7}	E41	I	1.15×10^{-6}
E9	S	1.53×10^{-4}	U43	I	8.22×10^{-5}
E10	S	5.88×10^{-4}	U51	I	2.84×10^{-6}
E13	I	5.45×10^{-6}	U51 *	I	1.40×10^{-5}
E14	I	1.48×10^{-5}	U66	S	7.22×10^{-4}
E15	S	3.68×10^{-7}	U71	I	3.12×10^{-4}
E21	S	6.02×10^{-7}	U80	I	3.89×10^{-6}

C = control, S = sensitive, I - intermediate

5.2.3 Minimum Inhibitory Concentrations of First Step Mutants

Table 5.2 shows the ciprofloxacin MICs (determined as described in Section 2.7) of the first-step mutants, selected from those generated by challenge of the parents with ciprofloxacin. Where there was an increase in the MIC of ciprofloxacin compared to the parent, the isolates were considered to be mutants. If there was no increase in the MIC of ciprofloxacin, they were disregarded for further work.

Table 5.2 MICs of selected first step mutants

ATCC19606	CIP MIC	* up MIC?	E13	CIP MIC	* up MIC?	E26	CIP MIC	* up MIC?	U51	CIP MIC	* up MIC?
Parent	**1**		**Parent**	**0.5**		**Parent**	**0.25**		**Parent**	**0.5**	
1	4	+	1	0.5	-	1	4	+	1	2	+
2	2	+	2	1	+	2	8	+	2	2	+
3	2	+	3	1	+	3	4	+	3	2	+
4	2	+	4	4	+	4	4	+	4	2	+
5	2	+	5	2	+	5	4	+	5	2	+
6	2	+	6	2	+	6	1	+	6	2	+
7	2	+	7	4	+	7	2	+	7	2	+
8	2	+	8	2	+	8	2	+	8	2	+
9	2	+									
ADP1			**E14**			**E33**			**U66**		
Parent	**0.06**		**Parent**	**0.5**		**Parent**	**0.12**		**Parent**	**0.03**	
1	0.5	+	1	8	+	1	1	+	1	0.25	+
2	0.5	+	2	4	+	2	0.25	+	2	0.12	+
3	0.5	+	3	2	+	3	0.5	+	3	0.25	+
4	0.5	+	4	4	+	4	1	+	4	0.25	+
5	0.5	+	5	4	+	5	1	+	5	0.12	+
6	0.5	+	6	4	+	6	1	+	6	0.12	+
7	0.5	+	7	2	+	7	1	+	7	0.25	+
8	0.5	+	8	8	+	8	1	+	8	0.12	+
9	0.5	+				9	0.5	+			
E9			**E15**			**E41**			**U71**		
Parent	**0.06**		**Parent**	**0.06**		**Parent**	**2**		**Parent**	**0.12**	
1	0.016	-	1	0.25	+	1	2	+	1	0.5	+
2	0.12	+	2	0.5	+	2	2	+	2	2	+
3	0.06	-	3	2	+	3	4	+	3	0.12	-
4	0.06	-	4	0.25	+	4	4	+	4	2	+
5	0.016	-	5	0.25	+	5	2	+	5	0.5	+
6	0.5	+	6	0.5	+	6	2	+	6	0.5	+
7	0.016	-	7	0.25	+	7	2	+	7	1	+
8	0.016	-	8	0.25	+	8	2	+	8	1	+
			9	0.5	+						
E10			**E21**			**U43**			**U80**		
Parent	**0.12**		**Parent**	**0.032**		**Parent**	**0.25**		**Parent**	**0.25**	
1	0.5	+	1	0.12	+	1	2	+	1	0.25	-
2	0.25	+	2	0.25	+	2	1	+	2	2	+
3	0.25	+	3	0.12	+	3	1	+	3	2	+
4	0.25	+	4	0.06	+	4	2	+	4	2	+
5	0.5	+	5	1	+	5	2	+	5	2	+
6	0.5	+	6	0.12	+	6	1	+	6	2	+
7	0.5	+	7	0.12	+	7	1	+	7	1	+
8	0.5	+	8	0.12	+	8	0.5	+	8	1	+
			9	0.12	+						

* up MIC – was the MIC increased compared to parent strain. + = yes, - = no.

It is apparent that there was a variation in the ranges of ciprofloxacin MICs in the potential mutants generated from different parental isolates. Those from parents with the R-type *mutS* sequence generally had higher ciprofloxacin MICs, up to 8 mg/L in two

mutants derived from gen.sp. 13TU isolate E14. This may be expected since the intermediate strains themselves had higher ciprofloxacin MICs than the sensitive parents, without the R-type *mutS* sequence. However, substantial increases in MIC levels were more frequent in 1st step mutants from parents with the R-type *mutS* sequence compared to the sensitive isolates without the R-type sequence.

Parent strains E9 and E10 (identified as gen.sp. 3 in Chapter 3) were noted above for their very high mutation frequencies, as shown in Table 5.1. However strain E9 produced only two mutants with higher ciprofloxacin MICs, and the increase in ciprofloxacin MICs in E10-derived mutants was not very high.

5.2.4 Generation of Second Step Mutants

Second-step mutants were generated as described in section 2.10.1 from selected first-step mutants which had various ciprofloxacin MICs. Mutation frequencies for the challenged first-step mutants, as a ratio of potential mutants to viable colonies, were determined as above and are displayed in Table 5.3.

Further mutants could not be generated from the 1st step mutants derived from parental isolates E9 and E10; there was no growth of E10-derived 1st step mutants on the 2 x ciprofloxacin MIC selective plates, and no viable growth of E9-derived 1st step mutants.

Table 5.3 Mutation frequencies of selected first-step mutants

Isolate	Mean mutation frequency	Isolate	Mean mutation frequency
ATCC 19606 (1)1	6.00×10^{-6}	E 26 / 7	2.38×10^{-3}
ATCC 19606 (3)10	1.63×10^{-5}	E26 / 8	7.30×10^{-7}
ADP1 (1)4	9.39×10^{-7}	E33 (2) 2	7.37×10^{-7}
ADP1 (2)6	1.13×10^{-6}	E33 (3) 1	5.88×10^{-7}
E13 / 2	2.60×10^{-4}	E41 / 3	1.38×10^{-6}
E14 / 2	5.10×10^{-4}	U43 / 1	2.77×10^{-2}
E14 / 4	6.77×10^{-2}	U51 (1) s1	7.40×10^{-7}
E14 / 8	1.31×10^{-4}	U51 (2) L1	6.53×10^{-7}
E15 (1) L2	3.20×10^{-7}	U66 / 3	3.20×10^{-6}
E15 (2) s3	5.00×10^{-9}	U71 / 4	2.17×10^{-6}
E21 (1) s5	2.07×10^{-6}	U80 / 3	2.53×10^{-5}
E21 (3) s2	3.42×10^{-6}		
E26 / 3	3.70×10^{-4}	U43+ / 4	2.50×10^{-6}
E 26 / 4	5.37×10^{-4}	U43+ / 6	8.36×10^{-4}

As observed for the parental strains, there was great variation in the mutation frequencies of the 1st step mutants, ranging here from 5×10^{-9} to 6.7×10^{-2}. Again the mutation frequencies of several isolates were very high, notably E14/4 with 6.7×10^{-2} and U43/1 with 2.7×10^{-2}. These were both 1st step mutants derived from intermediate parents (gen.sp. 13TU), with the R-type *mutS* sequence. The lowest mutation frequencies were found amongst the 1st step mutants derived from sensitive parents E15, E21, E26 and E33, as well as the non-clinical isolate ADP1. It appeared that there was a more obvious delineation between sensitive and intermediate isolates in terms of the mutation frequencies of their 1st step mutants compared to that of the isolates themselves.

5.2.5 Minimum Inhibitory Concentrations of Second Step Mutants

Ciprofloxacin MICs were established for the second-step mutants as described previously and those with elevated MICs compared to the first-step mutants were considered for further study. The ciprofloxacin MICs of the second-step mutants are shown in Table 5.4, with the data for parents also shown for comparison and to indicate the progression of ciprofloxacin resistance in each isolate. Generally there appeared to be less variation amongst MICs of 2nd step mutants than amongst 1st step mutants.

Table 5.4 Ciprofloxacin MICs of second-step *Acinetobacter* sp. mutants

Isolate	*	CIP MIC	Isolate	*	CIP MIC	Isolate	*	CIP MIC	Isolate	*	CIP MIC
ATCC 19606	**Parent**	**1**	**E14**	**Parent**	**0.5**	**E26**	**Parent**	**0.25**	**U43**	**Parent**	**0.25**
ATCC 19606 (1)1	**1st step**	**4**	**E14 / 4**	**1st step**	**4**	**E26 / 3**	**1st step**	**4**	**U43 / 1**	**1st step**	**2**
	(1) 1	4		1	16		1	32		1	4
	(1) 2	8		2	16		2	32		2	8
	(1) 3	8		3	16		3	32		3	4
ATCC 19606	**Parent**	**1**		4	16		4	16		4	4
ATCC 19606 (3)10	**1st step**	**4**		5	16		5	32		5	8
	(1)1	16		6	16		6	32		6	8
	(1)2	16	**E14**	**Parent**	**0.5**	**E26**	**Parent**	**0.25**	**U51**	**Parent**	**0.5**
	(1)3	16	**E14 /8**	**1st step**	**8**	**E26 / 4**	**1st step**	**4**	**U51(1)s1**	**1st step**	**2**
	(1)5	16		1	16		1	16		(1)1	8
	(2)1	16		2	16		2	32		(1)2	8
	(2)2	16		3	32		3	16		(1)3	8
	(2)3	16		4	128		4	16		(1)4	8
	(2)4	16		5	16		5	16		(1)5	8
	(2)5	16		6	16		6	16		(2)1	8
	(3)1	16	**E15**	**Parent**	**0.06**	**E26**	**Parent**	**0.25**		(2)2	8
	(3)2	16	**E15(1)L2**	**1st step**	**0.5**	**E26 / 7**	**1st step**	**2**		(2)3	8
	(3)3	16		(1)1	4		1	8		(2)4	8
	(3)4	16		(1)2	4		2	16		(2)5	8
	(3)5	16		(1)3	4		3	16		(3)1	32
ADP1	**Parent**	**0.06**		(1)4	4		4	8		(3)2	4
ADP1 (1)4	**1stStep**	**0.5**		(1)5	4		5	8		(3)3	8
	(1)1	2		(2)1	4		6	8		(3)4	8
	(1)2	2		(2)2	2	**E26**	**Parent**	**0.25**		(3)5	8
	(1)3	1		(2)3	2	**E26 / 8**	**1st step**	**2**	**U51**	**Parent**	**0.5**
	(2)1	2		(2)4	2		1	4	**U51(2)L1**	**1st step**	**2**
	(2)2	1		(2)5	2		2	8		(1)1	8
	(2)4	2		(3)1	4		3	8		(1)2	8
	(3)2	2		(3)2	4		4	16		(1)3	8
				(3)3	4		6	4		(1)4	8
				(3)4	2	**E33**	**Parent**	**0.12**		(1)5	8

Table 5.4 continued

Isolate		CIP MIC
	(3)3	2
	(3)4	2
	(3)5	2
ADP1	**Parent**	**0.06**
ADP1 (2)6	**1st step**	**0.5**
	(1)1	1
	(1)2	1
	(1)3	1
	(1)4	16
	(1)5	1
	(2)1	1
	(2)2	1
	(2)3	1
	(2)4	1
	(2)5	2
	(3)1	2
	(3)2	2
	(3)3	2
	(3)4	1
	(3)5	2
E13	**Parent**	**0.5**
E13 / 2	**1st step**	**1**
	1	1
	2	2
	4	4
	5	4
	6	4
E14	**Parent**	**0.5**
E14 / 2	**1st step**	**4**
	1	16
	2	16
	3	16
	4	32
	5	16
	6	32

Isolate		CIP MIC
	(3)5	2
E15	**Parent**	**0.06**
E15(2)s3	**1st step**	**0.25**
	(1)1	0.25
	(2)1	4
	(3)1	4
E21	**Parent**	**0.032**
E21(1)s5	**1st step**	**0.12**
	(1)1	1
	(1)2	1
	(1)3	0.5
	(1)4	1
	(1)5	2
	(2)1	1
	(2)2	1
	(2)3	0.5
	(2)4	1
	(2)5	2
	(3)1	1
	(3)3	1
	(3)4	1
	(3)5	1
E21	**Parent**	**0.032**
E21 (3)s2	**1st step**	**0.12**
	(1)1	1
	(1)2	1
	(1)3	1
	(1)5	0.5
	(2)1	1
	(2)2	1
	(2)3	1
	(2)4	1
	(2)5	1
	(3)1	1
	(3)3	1
	(3)4	1
	(3)5	1

Isolate		CIP MIC
E33(2)2	**1st step**	**1**
	(1)1	1
	(1)2	1
	(1)3	1
	(1)4	2
	(1)5	1
	(2)1	1
	(2)2	1
	(2)3	1
	(2)4	1
	(2)5	1
	(3)1	1
	(3)2	1
	(3)3	1
	(3)4	1
	(3)5	1
E33	**Parent**	**0.12**
E33(3)1	**1st step**	**1**
	(1)1	1
	(1)2	1
	(1)3	1
	(1)4	1
	(1)5	1
	(2)1	1
	(2)2	1
	(2)3	1
	(2)4	1
	(2)5	1
	(3)1	1
	(3)2	1
	(3)3	1
	(3)4	1
	(3)5	1
E41	**Parent**	**2**
E41 / 3	**1st step**	**4**
	1	8
	2	4
	3	8
	4	16
	5	16
	6	16

Isolate		CIP MIC
	(2)1	8
	(2)2	8
	(2)3	8
	(2)4	4
	(2)5	8
	(3)1	8
	(3)2	4
	(3)3	8
	(3)4	8
	(3)5	4
U66	**Parent**	**0.03**
U66 / 3	**1st step**	**0.25**
	1	1
	2	1
	3	1
	4	1
	5	1
	6	1
U71	**Parent**	**0.12**
U71 / 4	**1st step**	**2**
	1	4
	2	8
	3	4
	4	4
	5	4
	6	4
U80	**Parent**	**0.25**
U80 / 3	**1st step**	**2**
	1	4
	2	4
	3	4
	4	4
	5	4
	6	4

* Annotation for second step mutants shows individual mutants derived from the parent and 1st step mutant as shown. Bracketed numbers indicated their derivation from different plates inoculated with the 1st step mutant.

The high levels of ciprofloxacin MIC were observed most frequently in 2nd step mutants derived from intermediate parents with the R-type *mutS* sequence, with ciprofloxacin MICs of 8 and 16 mg/L common. There were also four occurrences of 32 mg/L ciprofloxacin MIC; one in an *A. baumannii* U51-derived 2nd step mutant, and three in gen.sp.13TU E14-derived 2nd step mutants. Additionally an E14-derived 2nd step mutant had a ciprofloxacin MIC of 128 mg/L.

The sensitive *Acinetobacter* spp isolates generally did not progress to 2nd step mutants with high ciprofloxacin MIC levels; the highest ciprofloxacin MIC in the majority of 2nd step mutants derived from sensitive isolates was 4mg/L, with 1mg/L being more common. For example, many of the 2nd step mutants derived from E33 for example did not have increased ciprofloxacin MICs compared to that of their 1st step mutant. The exception to this were the 2nd step mutants derived from E26, a sensitive *A. junii* isolate without the R-type *mutS* sequence, which developed ciprofloxacin MICs of 4 to 32 mg/L.

Second step mutants from the non-clinical strain ADP1, similarly to the sensitive isolates, did not have very high ciprofloxacin MIC levels, the majority being 1mg/L, although one 2nd step mutant did have a ciprofloxacin MIC of 16 mg/L.

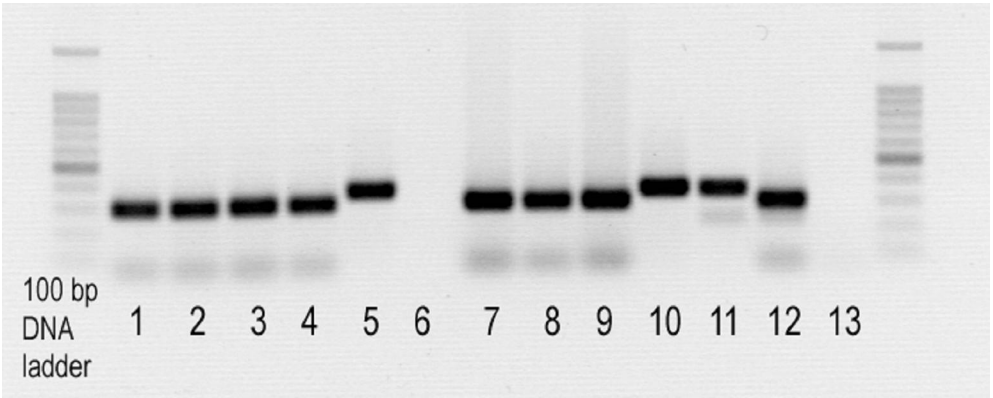
The greatest increase in ciprofloxacin MIC was observed in one of the 18 E14-derived 2nd step mutants. E14 is a gen.sp. 13TU intermediate isolate with the R-type *mutS* sequence. Parent to 1st step to 2nd step mutant ciprofloxacin MICs were 0.5 to 8 to 128 mg/L respectively, an increase of 256 x ciprofloxacin MIC of E14. Interestingly, the second greatest increase in ciprofloxacin MIC was observed in the non-clinical isolate ADP1, with a 250 x ciprofloxacin MIC increase with parent to 1st step to 2nd step mutant ciprofloxacin MICs of 0.06 to 0.5 to 16mg/L. However, this only occurred in one of the 25 ADP1-derived 2nd step mutants examined.

The lowest increase in ciprofloxacin MIC levels from parent to 2nd step mutants was observed in the sensitive isolate E33, with parent to 1st step to 2nd step mutant ciprofloxacin MICs of only 7 x ciprofloxacin MIC of E33.

5.2.6 *Hinf*I RFLP of the *gyrA* QRDR of parents and mutants

Target site mutations in the *gyrA* QRDR of a range of 1st and 2nd step mutants from each parental isolate were indicated by PCR amplification and *Hinf*I restriction, as described in Section 2.10.4. Isolates which have a mutation leading to loss of the *Hinf*I recognition site formed by codons 82 and 83 should produce a single product, visualised as one band of 343 bp on an agarose gel, compared to the restricted products which should be visualised as two bands of 291 and 54 bp. An example of a resultant gel of isolates U51, E14 and 1st and 2nd step mutants derived from them is shown in Figure 5.1.

Figure 5.1 Gel of *gyrA* restriction analysis of parent and mutants



Lane 1- U51 parent, CIP MIC 0.5mg/L. Lane 2 – U51-derived 1st step mutant, CIP MIC 2mg/L.
Lanes 3 to 5 - U51-derived 2nd step mutants, CIP MICs 4mg/L, 8mg/L and 32mg/L respectively.
Lane 6 – blank. Lane 7 – U71 parent, CIP MIC 0.12mg/L. Lane 8 – U71-derived 1st step mutant, CIP MIC 2mg/L.
Lanes 9 & 10 – U71-derived 2nd step mutants, CIP MICs 4 and 8mg/L respectively.
Lane 11 – Control – unrestricted ATCC 19606. Lane 12 – Control – restricted ATCC 19606.
Lane 13 – Control – DNA negative.

Table 5.5 shows the MIC data for the parents, 1st and 2nd step mutants, together with a summary of the range of second-step mutants derived from the different parental isolates and whether they were restricted or unrestricted.

Table 5.5 Progression to 2nd step mutants

	Parent	1st step	2nd step	R/UR
ATCC 19606	1 (R)	4 (R)	4	R
			8	R
		4 (R)	16	R
ADP1	0.06 (UR)	0.5 (UR)	1	UR
			2	UR
		0.5 (UR)	1	UR
			2	UR
			16	UR
E13	0.5 (R)	1 (R)	1	R
			2	R
			4	R
E14	0.5 (R)	4 (R)	16	R
			32	R
		4 (R)	16	R
		8 (R)	16	R
			32	R
			128	UR
E15	0.06 (R)	0.5 (R)	2	R
			4	R
		0.25 (R)	0.25	R
			4	-
E21	0.032 (R)	0.12 (R)	0.5	-
			1	R
			2	-
		0.12 (R)	0.5	R
			1	R

	Parent	1st step	2nd step	R/UR
E26	0.25 (R)	4 (R)	16	R
			32	R
		4 (R)	16	R
			32	R
		2 (R)	8	R
			16	R
		2 (R)	4	R
			8	R
			16	R
E33	0.12 (R)	1 (R)	1	R
			2	R
	0.12	1 (R)	1	-
E41	2 (R)	4 (R)	4	R
			8	R
			16	R
U43	0.25 (R)	2 (R)	4	R
			8	R
U51	0.5 (R)	2 (R)	4	R
			8	R
			32	UR
		2 (R)	4	-
			8	-
U66	0.03 (R)	0.25 (R)	1	-
U71	0.12 (R)	2 (R)	4	R
			8	UR
U80	0.25 (R)	2 (R)	4	R

R = g*yrA* PCR product restricted by *HinfI* – no target site mutation present.
UR = *gyrA* PCR product ununrestricted by *HinfI* – target site mutation may be present.
- = not tested.

Of immediate note from the *Hinf*I restriction analysis was that the ADP1 parent, 1st step and 2nd step mutants, were all unrestricted with *Hinf*I, regardless of ciprofloxacin MIC values, which ranged from parent to 1st step to 2nd step mutant from 0.06 to 0.5 to 1-16 mg/L respectively.

There were several unrestricted products, and hence potential target site mutations, in the 2nd step mutants. These all occurred in mutants derived from intermediate parents with the R-type *mutS* sequence: E14, U51 and U71 with unrestricted products in 2nd step mutants with ciprofloxacin MICs of 128, 32 and 8 mg/L respectively. There were other 2nd step mutants which also had higher ciprofloxacin MICs, notably those derived from the sensitive isolate E26, of which there were several with MICs of 16 and 32 mg/L. However there were no unrestricted products amongst these mutants.

5.2.7 Sequencing and Analysis of Parental and Mutant *gyrA* QRDRs

To further analyse the mutations that had led to the unrestricted products, the *gyrA* PCR products were sequenced as described in Section 2.10.5. The alignment of the sequenced *gyrA* QRDR of selected parents and mutants is shown in Figure 5.2 below, with the sequences compared to the ciprofloxacin sensitive strain HCP-77 (Vila *et al*, 1995).

It is apparent that whilst the *gyrA* QRDR appears highly conserved within these isolates, there is variation present within the isolates. The resistant isolates were included for comparison and, as expected, they have the serine-83 to leucine mutation (position 14 in Figure 5.2) in *gyrA* which has been commonly associated with ciprofloxacin resistance in *Acinetobacter*. HCP-77 is a ciprofloxacin susceptible *A. baumannii* strain, with the expected Serine at codon 83.

Figure 5.2 Sequences of the *gyrA* QRDR of selected parents and mutants compared to ciprofloxacin sensitive strain HCP-77 and resistant isolates

R = resistant isolate, I = intermediate parent isolate, S = sensitive parent isolate.
* positions where there was variation compared to strain HCP-77.
Bold indicates amino acid variations in the gyrA QRDR compared to strain HCP-77.

```
            1            *   *               *           *     50
HCP-77      VGDVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA Control
ATCC19606         KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA A.baumannii
ADP1              KYHP HGDTAVYDTI VRMAQDFSLR YLLVDGQGNF GSVDGDSAAA Non-clin
R U7           VIGKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA R
R E51            GKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA R
R 779          VIGKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA R
R 783          VIGKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA R
I E14             KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 0.5
  E14/8         GKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 8
  E14/8/4       GKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 128
S E21          IGKYHP HGDSAVYETI VRMAQDFSLR YQLVDGQGNF GSIDGDSAAA MIC = 0.032
S E26/4         GKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 4
  E26/4/2        KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 32
S E33               P HGDSAVYETI VRMAQDFSLR YQLVDGQGNF GSIDGDSAAA MIC = 0.12
I U43           GKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 0.25
  U43+/6        GKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 8
  U43+/6/4          P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 32
I U51               P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 0.5
  U51(1)s1       KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 2
  U51(1)s1(3) VIGKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 32
I U71           GKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 0.12
  U71/4          KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 2
  U71/4/2       GKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 8
I U80            KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 0.25
  U80/3         GEIHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 2
  U80/3/3       GKAHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA MIC = 4
                      *   *               *           *


            51      *   *                         * *    *   100
HCP77       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVP      Control
ATCC19606   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN A.baumannii
ADP1        MRYTEVRMTK LTHELLADLE KDTVDWVDNY DGSERIPDVL PTRIPNLLI  Non-clin
R U7        MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN R
R E51       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLI  R
R 779       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN R
R 783       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN R
I E14       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN MIC = 0.5
  E14/8     MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN MIC = 8
  E14/8/4   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN MIC = 128
S E21       MRYTEVRMRK LTHELLADLE KDTVDWEDNY DGSERIPQVM PTRIPNLL   MIC = 0.032
S E26/4     MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN MIC = 4
  E26/4/2   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLI  MIC = 32
S E33       MRYTEVRMRK LTHELLADLE KDTVDWEDNY DGSERIPQVM PTRIPNLL   MIC = 0.12
I U43       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN MIC = 0.25
  U43+/6    MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLI  MIC = 8
  U43+/6/4  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSKRIPEVL PTRVPNLLI  MIC = 32
I U51       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL            MIC = 0.5
  U51(1)s1  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL            MIC = 2
 U51(1)s1(3)1MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPN     MIC = 32
I U71       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIN MIC = 0.12
  U71/4     MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLI  MIC = 2
  U71/4/2   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLI  MIC = 8
I U80       MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLI  MIC = 0.25
  U80/3     MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPETL DTRSPNNLI  MIC = 2
  U80/3/3   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVPNLLIA MIC = 4
                  *   *                     *   * *
```

The sequences showed that the unrestricted products seen for ADP1 and derived mutants were not due to the frequently reported target site mutation of serine-83 to leucine. Instead at this position a mutation from serine to threonine was present, conserved in the parent and both first and second step mutants. As shown in Table 5.6 and Figure 5.3 below, this resulted from a nucleotide change from TCA to ACC, leading to loss of the *Hinf*I GANTC recognition site.

Table 5.6 Table of changes in the *gyrA* sequences of ADP1 and ADP1-derived mutants compared to HCP-77 and ATCC 19606.

				Changes with respect to HCP-77			
		CIP MIC	***Hinf*I R/UR**	**AA pos**	**AA change**	**Codon**	**Silent mutations**
ATCC 19606	Parental	1	R	None	-	-	3
	1st step mutants	4	R				
	2nd step mutants	8	R				
ADP1	Parental	0.06	UR	14 18 43 62 77 88 94	Ser – Thr Glu – Asp Ile – Val Ala – Thr Glu – Val Glu – Asp Val - Ile	TCA – ACC GAA – GAC ATC – GTC GCA – ACC GAA – GTA GAA – GAT GTT - ATT	33
	1st step mutants	0.5	UR				
	2nd step mutants	2	UR				

Additionally, there were a further 6 amino acid variations compared to HCP-77, again conserved in the parent and mutants of ADP1. None of these changes appeared to be associated with high ciprofloxacin MIC levels. There were also 33 silent mutations observed in the nucleotide sequence of ADP1, conserved in the 1st and 2nd step mutants derived from it. These changes are also detailed in Table 5.6 and Figure 5.3.

Figure 5.3 The *gyrA* QRDR of ADP1 and ADP1-derived 1st and 2nd step mutants compared to an *A. baumannii* quinolone-susceptible strain

HCP-77 = quinolone-susceptible strain used for comparison (Vila, 1995)
ADP1 par = ADP1 parent isolate
ADP1 1st = ADP1 1st step mutant ADP (1) 4
ADP1 2nd = ADP1 2nd step mutant ADP (1) 4 (1) 1
* indicates variation in ADP1 and ADP1-derived mutants compared to strain HCP-77
BOLD highlights changes compared to HCP-77.

Amino acid sequences:

```
          1              *   *                            *     50
HCP-77    VGDVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA
ADP1 par        KYHP HGDTAVYDTI VRMAQDFSLR YLLVDGQGNF GSVDGDSAAA   CIP MIC 0.06
ADP1 1st      GKYHP HGDTAVYDTI VRMAQDFSLR YLLVDGQGNF GSVDGDSAAA   CIP MIC 0.5
ADP1 2nd    VIGKYHP HGDTAVYDTI VRMAQDFSLR YLLVDGQGNF GSVDGDSAAA   CIP MIC 2
                       *   *                            *

          51           *               *            *      *   100
HCP-77    MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL PTRVP
ADP1 par MRYTEVRMTK LTHELLADLE KDTVDWVDNY DGSERIPDVL PTRIPNLLI    CIP MIC 0.06
ADP1 1st MRYTEVRMTK LTHELLADLE KDTVDWVDNY DGSERIPDVL PTRIPNLLI    CIP MIC 0.5
ADP1 2nd MRYTEVRMTK LTHELLADLE KDTVDWVDNY DGSERIPDVL PTRIPNLLIN   CIP MIC 2
                    *               *            *      *
```

Nucleotide sequences:

```
          1                                                      60
HCP-77    GTTGGTGACG TAATCGGTAA ATATCACCCG CATGGTGACT CAGCTGTTTA TGAAACCATT
ADP1 par                    AA ATATCACCCA CATGGTGATA CCGCTGTTTA CGACACGATC
ADP1 1st           G TGATCGGTAA ATATCACCCA CATGGTGATA CCGCTGTTTA CGACACGATC
ADP1 2nd          TG TGATCGGTAA ATATCACCCA CATGGTGATA CCGCTGTTTA CGACACGATC
          61                                                     120
HCP-77    GTTCGTATGG CTCAAGACTT TAGCTTACGT TATTTATTGG TTGATGGTCA GGGTAACTTC
ADP1 par GTGCGTATGG CGCAGGACTT CAGTCTGCGT TATCTATTGG TAGACGGTCA GGGCAACTTT
ADP1 1st GTGCGTATGG CGCAGGACTT CAGTCTGCGT TATCTATTGG TAGACGGTCA GGGCAACTTT
ADP1 2nd GTGCGTATGG CGCAGGACTT CAGTCTGCGT TATCTATTGG TAGACGGTCA GGGCAACTTT
          121                                                    180
HCP-77    GGTTCGATCG ATGGTGATAG CGCTGCGGCA ATGCGTTATA CCGAAGTCCG TATGACTAAG
ADP1 par GGTTCGGTCG ATGGCGATAG TGCTGCGGCA ATGCGTTATA CCGAAGTTCG TATGACTAAG
ADP1 1st GGTTCGGTCG ATGGCGATAG TGCTGCGGCA ATGCGTTATA CCGAAGTTCG TATGACTAAG
ADP1 2nd GGTTCGGTCG ATGGCGATAG TGCTGCGGCA ATGCGTTATA CCGAAGTTCG TATGACTAAG
          181                                                    240
HCP-77    CTGGCACATG AGCTTCTTGC AGATTTAGAA AAAGACACAG TTGACTGGGA AGATAACTAC
ADP1 par CTTACCCATG AGCTATTGGC TGATCTTGAA AAAGACACCG TAGACTGGGT AGATAACTAC
ADP1 1st CTTACCCATG AGCTATTGGC TGATCTTGAA AAAGACACCG TAGACTGGGT AGATAACTAC
ADP1 2nd CTTACCCATG AGCTATTGGC TGATCTTGAA AAAGACACCG TAGACTGGGT AGATAACTAC
          241                                                    300
HCP-77    GACGGTTCGG AACGTATCCC TGAAGTACTT CCGACACGAG TTCCA
ADP1 par GATGGTTCAG AACGTATTCC TGATGTTCTT CCGACACGCA TTCCAAATTT GCTGAT
ADP1 1st GATGGTTCAG AACGTATTCC TGATGTTCTT CCGACACGCA TTCCAAATTT GCTGATTAAC
ADP1 2nd GATGGTTCAG AACGTATTC
```

There were also several amino acid variations observed in E21 and E33 (both identified as *A. johnsonii* in Chapter 3) compared to HCP-77; six changes were conserved within the two isolates and the mutants generated from them. Again these changes were not associated with high ciprofloxacin MICs. Examination of the nucleotide sequences found that the changes were identical in the parents and mutants of both isolates at the nucleotide level, and also indicated the presence of 34 silent mutations in E21 and 30 silent mutations in E33. This is detailed in Table 5.7.

Table 5.7 Changes in *gyrA* sequences of E21 and E33 compared to HCP-77

		CIP MIC	HinfI R/UR	Changes wrt. HCP-77			
				AA pos	AA change	Nuc	Silent mutations
E21	Parental	0.032	R	32 59	Leu – Gln Thr – Arg	TTA –CAA ACT – CGT	34
	1st step mutants	0.12	R	62 88	Ala – Thr Glu – Gln	GCA – ACC GAA – CAA	
	2nd step mutants	1	R	90 94	Leu – Met Val – Ile	CTT – ATG GTT – ATT	
E33	Parental	0.12	R	32 59	Leu – Gln Thr – Arg	TTA –CAA ACT – CGT	30
	1st step mutants	1	R	62 88	Ala – Thr Glu – Gln	GCA – ACC GAA – CAA	
	2nd step mutants	2	R	90 94	Leu – Met Val – Ile	CTT – ATG GTT - ATT	

The previously mentioned 2nd step mutants from intermediate isolates that had unrestricted products (E14/8/4, U51(1)s1(3)1 and U71/4/2) were all confirmed to have the serine-83 to leucine target site mutation, as present in the resistant isolates, and this was associated in each case with a high ciprofloxacin MIC. The stabilities of each of

these mutants and their 1st step mutants were tested (as described in Section 2.10.2) and all were found to be stable within these conditions.

The sequences as observed in Figure 5.2 above showed that the parent and 1st step mutant of each 2nd step mutant that developed a target site mutation had a serine residue at codon 83, as present in the ciprofloxacin sensitive strain HCP-77. Figure 5.4 below illustrates this using U51 and mutants derived from U51 as an example.

Figure 5.4 Sequences of the *gyrA* QRDR of U51 and derived mutants with various ciprofloxacin MICs

HCP-77 = quinolone-susceptible strain used for comparison (Vila, 1995)
U51 par = U51 parent isolate
U51 1st = U51 1st step mutant U51(1)s1
U51 2nd = U51 2nd step mutants U51(1)s1(3)2, U51(1)s1(1)1 and U51(1)s1(3)1 with CIP MICs 4, 8 and 32 respectively.
* indicates aa position 83, where Ser83 – Leu target site mutation occurs
+ indicates codons 82 and 83, comprising the HinfI recognition site (GANTC).

```
        1             *                                    50
HCP-77  VGDVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA
U51 Par           P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 0.5
U51 1st       KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 2
U51 2nd       KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 4
U51 2nd       KYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 8
U51 2nd    VIGKYHP HGDLAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 32
                  *

                                                  82  83
        1                                         ++++ ++      50
HCP-77  GTTGGTGACG TAATCGGTAA ATATCACCCG CATGGTGACT CAGCTGTTTA
U51 Par                  AA ATATCACCCG CATGGTGACT CAGCTGTTTA CIP MIC = 0.5
U51 1st                  AA ATATCACCCG CATGGTGACT CAGCTGTTTA CIP MIC = 2
U51 2nd        ACG TAATCGG-AA ATATCACCCG CATGGTGACT CAGCTGTTTA CIP MIC = 4
U51 2nd                               CATGGTGACT CAGCTGTTTA CIP MIC = 8
U51 2nd         CG TAATCGGTAA ATATCACCCG CATGGTGACT TAGCTGTTTA CIP MIC = 32
                                         ++++ ++
```

The sequences showed that U51 and the 1st step mutant, as well as two 2nd step mutants with ciprofloxacin MICs of 4 and 8 mg/L had serine at position 83. However the 2nd step mutant with MIC of 32 mg/L had the serine-83 to leucine target site mutation, occurring from a C-T nucleotide substitution. There were no other mutations present in either the amino acid or nucleotide sequence of the isolate, as is observed in the resistant isolates.

Also of note from the sequence analysis of the *gyrA* QRDR of the parents and mutants is that a 2nd step mutant, from a 1st step mutant which was derived from the U43 isolate challenged with 20 x ciprofloxacin MIC, had a mutation at codon 153, from glutamic acid to lysine. This is examined, along with other aspects of the U43+ isolates in Section 5.2.8 below.

5.2.8 U43+ Mutants

These mutants were derived from challenge by 20 x MIC of ciprofloxacin, and were the only isolates out of those so challenged from which mutants grew. The colonies produced by the initial challenge were large and mucoid in appearance, in contrast to those seen with mutants produced by challenge with 2 x MIC of ciprofloxacin.

The U43+ mutants were stored similarly to the normally challenged isolates and used to generate 2nd step mutants (by challenge at 2 x MIC). This procedure was also repeated with a confirmed amount of ciprofloxacin; U43 was challenged by 10 x MIC of

ciprofloxacin, and 1st step mutants (labelled U43x10) by 2 x MIC to give 2nd step mutants. The MICs of these U43 derived mutants are shown in Table 5.8.

Table 5.8 MIC (mg/L) progression of U43 mutants challenged by excess ciprofloxacin

	CIP MIC		CIP MIC		CIP MIC
1st step mutants generated by challenge with excess ciprofloxacin					
U43 parent	0.25	U43+/4	4	U43+/6	8
1st step mutants:		2nd step mutants:		2nd step mutants:	
U43+/1	2	U43+/4/1	4	U43+/6/1	32
U43+/2	2	U43+/4/2	8	U43+/6/2	32
U43+/3	2	U43+/4/3	8	U43+/6/3	32
U43+/4	4	U43+/4/4	8	U43+/6/4	32
U43+/5	4	U43+/4/5	8	U43+/6/5	16
U43+/6	8	U43+/4/6	8	U43+/6/6	32
1st step mutants generated by challenge with 10x MIC of ciprofloxacin					
U43 parent	0.25				
1st step mutants:					
U43x10/1	8	U43x10/11	8	U43x10/21	8
U43x10/2	8	U43x10/12	16	U43x10/22	8
U43x10/3	8	U43x10/13	16	U43x10/23	8
U43x10/4	8	U43x10/14	16	U43x10/24	8
U43x10/5	8	U43x10/15	8	U43x10/25	16
U43x10/6	8	U43x10/16	2	U43x10/26	16
U43x10/7	8	U43x10/17	8	U43x10/27	8
U43x10/8	8	U43x10/18	4	U43x10/28	8
U43x10/9	16	U43x10/19	8	U43x10/29	16
U43x10/10	2	U43x10/20	8	U43x10/30	16
2nd step mutants:					
U43x10/7/1	32	U43x10/26/1	128		
U43x10/7/2	64	U43x10/26/1	64		
U43x10/7/3	32	U43x10/26/1	128		
U43x10/7/4	32	U43x10/26/1	64		
U43x10/7/5	32	U43x10/26/1	128		
U43x10/7/6	32	U43x10/26/1	128		
U43x10/7/7	32	U43x10/26/1	64		
U43x10/7/8	32	U43x10/26/1	128		

As is shown in the data above, the second challenge (with 10 x MIC of ciprofloxacin) generated greater increases in ciprofloxacin MICs of 1st and subsequent 2nd step mutants, up to 128 mg/L.

As mentioned above, the *gyrA* sequence analysis of the initially derived U43+ mutants showed a glutamic acid-153 to lysine mutation in the *gyrA* QRDR of several 2nd step mutants, shown in Figure 5.5 below. This mutation did appear to be associated with a higher ciprofloxacin MIC level of 32 mg/L, compared to the other 2nd step mutant with a ciprofloxacin MIC of 8 mg/L. The stability of the U43+/6/4 mutant was tested (as described in Section 2.10.2) and found to be stable within these conditions.

Figure 5.5 *gyrA* sequences of U43 and initial U43+ isolates compared to HCP-77

HCP-77 = quinolone-susceptible strain used for comparison (Vila, 1995)
U43 par = U43 parent isolate
U43/1 = U43 1st step mutant
U43/1/1 = U43 2nd step mutant
U43+/6 = U43 1st step mutant from challenge with excess ciprofloxacin
U43+/6/3 and U43+/6/4 = U43+ 2nd step mutants derived from the above 1st step
* = position where there is a change compared to HCP-77.

```
         1                                                  50
HCP-77   VGDVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA
U43 par    DVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 0.25
U43/1      RVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 2
U43/1/1    DVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 4
U43+/6        GKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 8
U43+/6/3   RVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 32
U43+/6/4         P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 32

         51                                  *    90
HCP-77   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL
U43 par  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL  CIP MIC = 0.25
U43/1    MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL  CIP MIC = 2
U43/1/1  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL  CIP MIC = 4
U43+/6   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL  CIP MIC = 8
U43+/6/3 MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSKRIPEVL  CIP MIC = 32
U43+/6/4 MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSKRIPEVL  CIP MIC = 32
                                            *
```

However, this mutation was not found in sequence analysis of the 2nd step U43x10 mutants, with MICs of 32 to 128 mg/L. This is shown in Figure 5.6 below.

Figure 5.6 *gyrA* sequences of further U43+ compared to previously generated mutant and HCP-77

HCP-77 = quinolone-susceptible strain used for comparison (Vila, 1995)
U43+/6/4 = U43 2nd step mutant derived from challenge of U43 with excess CIP 1st step
U43+/7 and U43+/26 = 1st step mutants generated by challenge with 10 x MIC CIP of U43
U43+/7/1, U43+/7/2, U43+/26/6 and U43+/26/7 = 2nd step mutants generated by challenge of above with CIP
* = position where there is a change compared to HCP-77.

```
          1                                                  50
HCP-77    VGDVIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA
U43+/6/4            P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 32
U43+/7         VIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 8
U43+/26        VIGKYHP HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 16
U43+/7/1            P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 32
U43+/7/2            P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 64
U43+/26/6           P HGDSAVYETI VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 128
U43+/26/7                        VRMAQDFSLR YLLVDGQGNF GSIDGDSAAA CIP MIC = 64

          51                                    *    90
HCP-77    MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL
U43+/6/4  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSKRIPEVL CIP MIC = 32
U43+/7    MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL CIP MIC = 8
U43+/26   MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL CIP MIC = 16
U43+/7/1  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL CIP MIC = 32
U43+/7/2  MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL CIP MIC = 64
U43+/26/6 MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL CIP MIC = 128
U43+/26/7 MRYTEVRMTK LAHELLADLE KDTVDWEDNY DGSERIPEVL CIP MIC = 64
                                      *
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Despite the inability to replicate the amino acid change observed in the initial 2nd step mutants from U43 challenged with excess ciprofloxacin, the stability of this mutant may mean that it warrants further investigation.

5.2.9 Mutation Studies Summary

Mutation frequencies of the representative isolates and 1st step mutants derived from them showed great variation. Amongst the highest mutation frequencies of the parent isolates were E9 and E10, however 1st step mutants generated from them were not able

to go on to develop 2nd step mutants. The highest mutation frequencies of the 1st step mutants were in those derived from intermediate isolates with the R-type *mutS* sequence and the lowest was in 1st step mutants derived from sensitive isolates and ADP1.

Substantial increases in ciprofloxacin MICs were more frequent in 1st step mutants derived from isolates with the R-type *mutS* sequence compared to those without this sequence. There was a smaller range of ciprofloxacin MIC values generally in 2nd step mutants compared to the 1st step mutants, and isolates without the *mutS* R-type sequence generally didn't progress to high ciprofloxacin MIC levels. The greatest increase in ciprofloxacin MICs was observed in a 2nd step mutant from isolate E14 (from parent to 2nd step, 0.5 to 128mg/L), and the lowest were observed in 2nd step mutants from isolate E33 (from parent to 2nd step, 0.12 to 1mg/L).

Target site mutations of Ser83-Leu were confirmed to be present in representative resistant isolates and were found in one 2nd step mutant derived from each of E14 (gen.sp. 13TU), U51 (*A. baumannii*) and U71 (*A. baumannii*), with ciprofloxacin MICs of 128, 32 and 8 mg/L respectively.

The non-clinical *A. baylyi* isolate ADP1 and all mutants derived from it all had mutations at serine-83, however sequencing confirmed that this was a conserved serine to threonine mutation, and it did not appear to be associated with high ciprofloxacin MICs.

U43+ and U43x10 mutants, challenged with 20 x MIC and 10 x MIC respectively, developed high ciprofloxacin MICs, and several had an associated glutamic acid 153 to lysine *gyrA* mutation.

5.3 Discussion

The question posed at the beginning of this chapter was whether the different *mutS* sequence types discussed in Chapter 4 were correlated with any differences in the mutation potential of isolates, in terms of their ability to develop increased resistance to ciprofloxacin. It is clear from the above data that there is variation in the mutation frequencies of the clinical *Acinetobacter* spp isolates, and in their ability to develop significant ciprofloxacin resistance upon challenge.

The varied mutation frequencies of parental isolates did not clearly correlate with *mutS* type, though the lowest mutation frequencies for both parents and 1st step mutants were observed in those from sensitive, non-R-type *mutS* isolates. There did appear to be a correlation of high mutation frequencies of 1st step mutants in those derived from parents with R-type *mutS*. The sensitive *A. junii* isolate E26 was an exception to this however, with a high mutation frequency of parent and several 1st step mutants compared to the other sensitive isolates, and also to several intermediate isolates. Additionally, high ciprofloxacin MICs were observed in 2nd step mutants derived from this isolate, though none possessed a corresponding *gyrA* target site mutation.

The variation in mutation frequencies does show that there may be certain hypermutator isolates that are better able to adapt to ciprofloxacin challenge by increasing their chance of producing beneficial mutations and of acquiring resistance through horizontal transfer (as discussed in Section 1.5). However it must be remembered that this is a measure of spontaneous mutation frequency, and as such the resultant mutant may not in fact be stable, nor lead to clinically significant resistance (Section 1.5; O'Neill & Chopra, 2002; Martinez & Baquero, 2000; Woodford & Ellington, 2007). Also of note is that reported bacterial mutation frequencies vary greatly between studies, possibly due to the complexity and variation of the environments (Woodford & Ellington, 2007) so these figures alone may not be a reliable measure of mutation potential, and it is very difficult to compare mutation frequencies between studies. Therefore, there should be caution when examining mutation frequencies in isolation as, whilst they give an indication of the potential of mutations to develop, a high mutation frequency does not guarantee the production of beneficial mutations in viable mutants.

This is likely the case in the sensitive gen.sp. 3 isolates E9 and E10. These isolates had the highest mutation frequencies observed in the parental strains; however the lack of increase in ciprofloxacin MIC in potential mutants derived from E9, and the inability to generate 2nd step mutants from either E9 or E10, confirms that this did not manifest as a greater mutation potential. The 'mutants' produced and measured in the mutation frequency calculation were clearly not viable in the longer term. This supports the caution about the use of mutation frequency data in isolation to establish which isolates

will have greater mutation potential; whilst a higher mutation frequency may increase the chances of a useful mutation developing, it may also increase the risk of deleterious mutations and render the bacterium unviable in the longer term.

In terms of the *gyrA* gene of initial interest was whether any of the 2nd step mutants had the *gyrA* Ser83-Leu target site mutation that was confirmed to be present in the resistant isolates. Within the data collected *gyrA* target site mutations were only observed in several 2nd step mutants derived from intermediate parents with the R-type *mutS* sequence (*A. baumannii* isolates U51 and U71, and gen.sp. 13TU isolate E14). These were associated with high ciprofloxacin MICs and were found to be stable in the absence of antibiotic selective pressure (Section 2.10.2).

Whilst there were other 2nd step mutants with high ciprofloxacin MICs, including those derived from sensitive parents without the R-type *mutS* sequence, these did not have *gyrA* target site mutations; for example E26, a sensitive *A. junii* isolate which had several variations compared to the R-type *mutS* sequence (Chapter 4), had a high mutation frequency and several of its 1st step mutants also had a high mutation frequency in response to challenge with ciprofloxacin. In comparison with the other sensitive isolates E9 and E10 discussed above, the 2nd step mutants of this isolate had high ciprofloxacin MICs and, although producing no target site mutations, this suggests that this isolate may have a greater mutation potential than the other sensitive isolates.

This may be attributable to the over-expression of efflux mechanisms, increasingly considered an important secondary fluoroquinolone resistance mechanism. The AdeB multidrug efflux pump in *A. baumannii*, for example, is responsible for aminoglycoside resistance and also associated with reduced susceptibility to fluoroquinolones, although other mechanisms (target site mutations) are needed for resistance to build up (Section 1.4.6; Higgins *et al*, 2004). It is thought that efflux is of importance in the first response to challenge with antibiotic, allowing initial survival and hence increasing the chance of the development of stable and lasting resistance via target site mutations (Section 1.4; Poole, 2002; Van Bembeke, 2005). This may be the case in the 2nd step mutants derived from E26; that efflux is over-expressed in response to the challenge with ciprofloxacin, temporarily raising the ciprofloxacin MICs as the antibiotic is pumped out of the bacterial cells, but without production of target site mutations.

However, resistance to fluoroquinolones in *Acinetobacter* isolates is primarily via the target site mutations in *gyrA* genes (Section 1.4.6; Vila *et al*, 1995; Magnet *et al*, 2001; Higgins *et al*, 2004), as was confirmed in the resistant and multi-resistant isolates in this study which had the *gyrA* Ser83-Leu target site mutation (Figure 5.2). It would, therefore, be expected that emerging-resistant isolates would develop clinically significant resistance via similar methods. As such, it is reasonable to conclude that those isolates producing 2nd step mutants with high ciprofloxacin MICs and *gyrA* target site mutations (in this study, those that were derived from intermediate parents with the R-type *mutS* sequence) have a greater mutation potential than those with high

ciprofloxacin MICs but without concurrent target site mutations (in this study, those derived from sensitive isolate E26, without the R-type *mutS* sequence).

In terms of *gyrA* gene variation beyond the observed target site mutations, the intermediate isolate-derived 2nd step mutants with target site mutations displayed only this Ser-83 to Leu mutation within their *gyrA* gene. Additionally, there were no mutations within the parent and 1st step mutants of these 2nd step mutants, compared to the sequences of the ciprofloxacin-sensitive strain HCP-77. There were, however, other *gyrA* variations observed in several of the sequenced sensitive strains and derived mutants and in the non-clinical isolate ADP1 and derived mutants, none of which were associated with high MIC levels. These may be species-specific differences as E21 and E33 (in which there were several differences in *gyrA* amino acid sequence) were both identified as *A. johnsonii* (Chapter 3) and they are being compared to an *A. baumannii* ciprofloxacin-sensitive strain (HCP-77).

Additionally, this variation in the *gyrA* gene of clinical isolates is of interest; whilst the *gyrA* gene of the examined resistant and intermediate isolates and derived mutants was highly conserved, that of the sensitive and non-clinical isolates was more varied. Likewise the ciprofloxacin MICs of successful 2nd step mutants were less varied than those seen for 1st step mutants (Figures 5.2 and 5.4). This may be a similar observation as in Chapter 4 in terms of the *mutS* gene; that with increasing resistance, there is greater homogeneity.

The *gyrA* QRDR of ADP1 and all mutants derived from it were unrestricted with *Hinf*I. Sequence analysis showed that instead of being due to the serine-83 to leucine mutation, the loss of restriction site was due to a serine-83 to threonine mutation. Serine and threonine are both polar amino acids, so it may be that this mutation has less effect than on the fluoroquinolone target site, and hence less impact on ciprofloxacin resistance, when compared to the change from polar to hydrophobic amino acid (leucine) at the same site within the *gyrA* QRDR. This result highlights the need for sequencing confirmation of initial restriction analysis results, to verify the nature of the mutation causing the unrestricted product; this agrees with previous reports where mutation at the target site, leading to PCR products unrestricted by *Hinf*I, was not associated with higher MICs (Waters & Davies, 1997).

The U43 mutants produced from challenge with excess ciprofloxacin (10 x MIC and 20 x MIC) developed high ciprofloxacin MICs, but no target site mutations. Increased MICs resulting from a greater challenge is perhaps to be expected, however the ability of U43 alone of the examined isolates to respond to this challenge is worth noting. The glutamic acid-153 to lysine mutation seen in two of the 2nd step mutants was not reproducible, and is therefore unlikely to be the cause of the increase in ciprofloxacin MIC. It may be that the increase in MIC is due to efflux over-expression, as discussed in Section 1.4.6 and above; however, the mutants were stable in non-selective media and the *gyrA* mutation was the only one present in the *gyrA* QRDR, so the association with high MIC levels may warrant further investigation. Given the mucoid appearance of the

excessively-challenged mutants, there is a possibility that biofilm formation was activated by this excess ciprofloxacin challenge.

Several studies report a high capacity of multiresistant *A. baumannii* clinical isolates to form biofilms (Lee *et al*, 2008; Rodríguez-Baño *et al*, 2008), and different morphologies have been reported in low and high nutrient conditions which are hypothesised to represent specialised adaptations for, respectively, attachment and colonisation or dispersion. (James, 1995). Environmental and clinical members of the *Acinetobacter* genus have been reported to attach to solid surfaces and form biofilms which allow them to persist in harsh conditions, associated with the presence of pili-like surface structures encoded by the *csuC* and *csuE* ORFs (Tomaras *et al*, 2003). It may be that the extreme ciprofloxacin challenge provoked similar adaptation and the initiation of biofilm formation in the U43+ mutants generated in this study, though of course further investigation would be needed to confirm this theory.

In terms of the species of the *Acinetobacter* genus capable of ciprofloxacin resistance development, it is interesting to note that not only *A. baumannii* isolates (U51 and U71), but also a gen.sp. 13TU isolate (E14) was capable of developing significant ciprofloxacin resistance in terms of a target site mutation; indeed generally the 2nd step mutants derived from gen.sp. 13TU parents, compared to those from *A. baumannii* parents, developed higher ciprofloxacin MICs (Figure 5.5). This included the aforementioned 2nd step mutant with ciprofloxacin MIC of 128mg/L, which also had a

gyrA target site mutation (derived from gen.sp. 13TU isolate E14). This ability of gen.sp. 13TU agrees with the increasingly common consensus that gen.sp. 13TU is also of importance clinically (Section 1.3; Dijkshoorn *et al*, 2007). Additionally gen.sp. 13TU isolate U43 developed high ciprofloxacin MICs upon challenge with 10 x MIC, and indeed was the only isolate able to survive this high challenge. Furthermore *A. junii* (E26)-derived 2nd step mutants, as mentioned above, also developed high levels of ciprofloxacin MIC. Whilst these raised levels alone may not be indicative of clinically significant resistance, unlike the development of target site mutation, survival of this challenge may allow these isolates to be more successful in the clinical environment and with further challenges they could go on to develop clinical significant target site mutations. This therefore raises questions about whether further *Acinetobacter* species, and not just those of the Acb complex, may be more relevant in the clinical situation than thought. Of importance is that the sensitive *A. baumannii* isolate without the R-type *mutS* amino acid sequence (U66) did not develop mutants with high ciprofloxacin MICs, nor *gyrA* target site mutations, agreeing with the suggestion that the ability to develop resistance, whilst common in *A. baumannii* as discussed in Section 1.3, may not be due to species alone.

Regarding fluoroquinolone resistance itself, as opposed to its use as a tool in this study, the ability of *Acinetobacter* spp to readily develop high levels of resistance upon challenge with ciprofloxacin agrees with the observation that the rise in fluoroquinolone resistance is particularly dramatic (Livermore, 2007). As mentioned (Section 1.4.4),

fluoroquinolone resistance is a risk factor for epidemic behaviour in *Acinetobacter* (Koeleman, 2001) and is associated with outbreak strains of *Acinetobacter* (Wisplinghoff, 2003; Heinemann *et al*, 2000), therefore the ability of not only *A .baumannii* but also other *Acinetobacter* spp to readily develop high levels of ciprofloxacin resistance is of concern.

In conclusion, from the examined clinical *Acinetobacter* isolates it appears that those isolates with the R-type *mutS* sequence delineated in Chapter 4 appeared more capable of following the fluoroquinolone resistance development pathway seen in the resistant and multi-resistant isolates, with the development of *gyrA* target site mutations and resultant increases in MICs. In species terms, these comprised two *A. baumannii* and one gen.sp. 13TU isolate. Sensitive and intermediate isolates with variations to the R-type sequence in comparison did not seem as capable of developing such resistance to ciprofloxacin, with no target site mutations observed in any mutants derived from these isolates, and less frequent occurrence of high ciprofloxacin MICs. Taken together, these observations suggest that emerging-resistant isolates become more similar as resistance develops, and support the hypothesis that genetically distinct sub-populations of *Acinetobacter* spp may exist in the clinical environment, with differences in the *mutS* gene and an increased ability to rapidly acquire antibiotic resistance.

Chapter 6: The efficacy and effects of Chlorhexidine upon MRSA

6.1 Introduction

This examination of the effects of chlorhexidine upon MRSA formed part of a project which also investigated the frequency of biocide resistance genes within the sample population of clinical MRSA (Vali *et al*, 2008; Appendix 1).

As discussed in more detail in Section 1.2, MRSA is of great concern in the clinical environment with growing prevalence and resistance levels leading to resurgence in infection control measures as a means to limit transmission and an alternative to reliance upon antibiotics to treat infections (Amyes, 2005; Grayson *et al*, 2008). With this has come a rise in the use of biocides, increasingly incorporated into products in both the clinical and domestic environments. This increase has led to concerns about use and misuse leading to reduced biocide susceptibility, with the possibility of cross-resistance to antibiotics, and concern regarding the efficacy of biocides upon clinical isolates as opposed to the tested standard strains (Russell *et al*, 1998; Levy, 2001). Whilst the in-use concentration is high it is thought that in practice low concentrations of biocide may be present, from misuse leading to diluted fluids or due to the presence of residues in the hospital environment (Bloomfield, 2002). As such the study of low concentration of biocides upon clinical isolates is increasingly advocated, and there is a paucity of information on this aspect at present (Maillard, 2007). Additionally, there is concern that the tested standard strains may not adequately reflect the efficacy of biocides against

current clinical isolates (Kampf *et al*, 1998). In particular the reliance upon infection control to counteract the spread of MRSA makes the importance of early detection of reduced susceptibility to biocides in these bacteria especially important.

MICs are generally considered insufficient for the testing of biocide reduced susceptibility and there is a lack of internationally standardised alternative methods (Section 1.2; Cookson, 2005). However several methods have been developed by Thomas and colleagues: a suspension test, surface disinfectant test and biocide residue test (Thomas *et al*, 2005), which were adapted for use here (Section 2.11).

As detailed in Section 1.6.6, this study aimed to evaluate the efficacy of chlorhexidine on current clinical isolates compared to control strains, and to investigate whether there is any correlation between exposure to chlorhexidine and the development of antibiotic resistance in common strains of MRSA.

6.2 Results

6.2.1 Isolate Characterisation

All isolates in this study (except the control *Acinetobacter baumannii* standard strain ATCC 19606 and the control sensitive *Staphylococcus aureus* strain NCTC 6571), were collected from the New Royal Infirmary Edinburgh (NRIE) and subsequently tested positive for the presence of the *mecA* gene, as determined by PCR (described in Section 2.6.2), confirming that they were MRSA. Control strains used were the aforementioned

S. aureus NCTC 6571 (S) and *A. baumannii* ATCC 19606 (A), and also EMRSA-16 (C).

6.2.2 Susceptibility of Isolates

The MICs determined for the 120 confirmed hospital-acquired MRSA isolates are shown in Table 6.1. All of the isolates were susceptible to vancomycin (breakpoint > 8 mg/L). Only 3.3% were resistant to tetracycline (breakpoint > 1 mg/L) and 27.5% to gentamicin (breakpoint > 1 mg/L). There was 89.2% resistance to oxacillin (breakpoint > 2 mg/L), 90% to cefuroxime (breakpoint > 4 mg/L), 96.7 to ciprofloxacin (breakpoint > 1 mg/L) and 98.3% to cefotaxime (breakpoint > 4 mg/L). The highest level of resistance was observed to ampicillin (breakpoint > 2 mg/L), with 99.2% of the isolates resistant.

Table 6.1 MICs of eight antibiotics to MRSA isolates

Isolate		Minimum Inhibitory Concentration (mg/L)							
		AMP	GEN	OXA	CTX	CEF	CIP	TET	VAN
LF	**1**	32	0.5	64	128	>128	64	0.25	1
LF	**2**	32	0.25	64	128	>128	64	0.25	0.5
LF	**3**	32	32	64	128	>128	64	0.25	0.5
LF	**4**	32	32	>128	>128	>128	64	0.25	0.5
LF	**5**	16	32	>128	>128	>128	128	32	0.5
LF	**6**	32	0.25	64	128	>128	64	0.25	0.5
LF	**7**	32	0.25	>128	>128	>128	128	0.25	1
LF	**8**	32	0.25	64	128	>128	32	0.25	1
LF	**9**	64	0.5	128	>128	>128	64	0.25	0.5
LF	**10**	32	0.25	64	>128	>128	128	0.25	0.5
LF	**11**	32	0.25	>128	>128	>128	64	0.25	0.5
LF	**12**	64	8	>128	>128	>128	128	0.25	0.5
LF	**13**	64	0.5	128	>128	>128	64	0.25	1
LF	**14**	32	32	32	64	>128	64	0.25	0.5
LF	**15**	16	0.25	8	16	64	128	0.25	0.5
LF	**16**	16	64	>128	>128	>128	64	0.25	0.5
LF	**17**	64	32	>128	>128	>128	64	0.25	1
LF	**18**	64	64	>128	>128	>128	64	0.25	0.5
LF	**19**	64	0.5	16	32	128	>128	0.25	1
LF	**20**	32	0.25	16	64	128	128	0.25	0.5
LF	**21**	32	32	64	64	128	64	0.25	0.5

Table 6.1 continued		MIC (mg/L)							
		AMP	GEN	OXA	CTX	CEF	CIP	TET	VAN
LF	22	16	0.25	16	64	64	128	0.25	1
LF	23	32	0.5	64	128	>128	16	0.25	1
LF	24	32	0.5	64	128	>128	64	0.25	0.5
LF	25	32	0.12	32	32	128	32	0.25	0.5
LF	26	32	0.25	32	128	>128	64	0.25	1
LF	27	32	0.12	128	128	>128	64	16	1
LF	28	32	0.12	32	64	>128	64	0.25	0.5
LF	29	32	0.12	16	64	128	16	0.25	0.5
LF	30	32	0.032	0.12	16	0.5	64	0.12	0.5
LF	31	64	16	128	>128	1	32	0.12	0.5
LF	32	32	32	32	32	128	32	0.25	0.5
LF	33	32	16	32	32	128	64	0.25	0.5
LF	34	16	32	128	>128	>128	64	0.25	0.5
LF	35	32	0.12	32	32	>128	16	0.25	0.5
LF	36	16	0.25	8	32	16	64	0.25	0.5
LF	37	32	0.02	0.002	32	0.004	64	0.06	0.02
LF	38	64	32	128	>128	>128	64	0.25	0.5
LF	39	32	0.12	16	32	32	64	0.25	0.5
LF	40	64	32	128	>128	>128	64	0.25	0.5
LF	41	16	32	128	>128	>128	64	0.25	0.5
LF	42	64	>128	>128	>128	>128	64	>128	1
LF	43	64	16	128	>128	>128	32	0.25	0.5
LF	44	64	0.12	64	32	>128	64	0.25	0.5
LF	45	32	0.5	64	128	>128	32	0.25	0.5
LF	46	32	0.12	16	32	128	64	0.5	0.5
LF	47	16	32	32	>128	>128	64	0.25	0.5
LF	48	32	0.002	0.004	16	0.5	64	0.12	0.25
LF	49	32	0.12	2	16	16	32	0.25	0.5
LF	50	64	32	>128	>128	>128	64	0.5	0.5
LF	51	32	0.002	0.12	128	0.25	64	0.12	0.25
LF	52	32	0.002	0.002	8	0.002	64	0.002	0.002
LF	53	64	0.002	0.12	>128	0.25	0.004	0.12	0.5
LF	54	64	0.002	0.12	>128	0.25	64	0.12	0.25
LF	55	32	32	32	32	128	64	0.25	0.5
LF	56	32	0.25	32	32	128	64	0.25	1
LF	57	64	0.002	0.12	>128	0.25	64	0.25	0.5
LF	58	32	0.002	0.12	32	0.25	64	0.25	0.5
LF	59	0.032	0.002	0.002	2	0.002	64	0.002	0.002
LF	60	16	0.25	32	64	32	64	0.5	1
LF	61	64	32	128	>128	>128	128	0.5	1
LF	62	64	32	128	>128	>128	64	0.5	1
LF	63	16	32	128	>128	>128	128	0.25	1
LF	64	32	0.25	32	16	64	128	0.5	1
LF	65	16	0.5	1	8	8	0.5	0.5	2
LF	66	32	32	16	16	64	64	0.25	1
LF	67	1	0.25	0.5	4	8	1	32	2
LF	68	64	32	128	>128	>128	64	0.25	1
LF	69	16	0.25	32	64	64	128	0.5	2
LF	70	32	0.25	32	128	128	128	0.5	2
LF	71	16	0.5	32	64	64	16	0.25	2
LF	72	16	0.25	16	64	32	64	0.25	1
LF	73	16	0.25	16	16	32	16	0.25	1
LF	74	16	32	128	>128	>128	128	32	1
LF	75	16	0.25	32	128	64	64	0.5	2
LF	76	16	32	>128	>128	>128	128	0.5	2
LF	77	16	0.5	8	64	32	64	0.25	1
LF	78	32	1	64	128	>128	64	0.25	2
LF	79	16	0.5	32	128	>128	>128	0.25	1
LF	80	128	0.5	>128	>128	>128	128	0.5	2
LF	81	64	32	>128	>128	>128	128	0.5	2

Table 6.1 continued		MIC (mg/L)							
		AMP	**GEN**	**OXA**	**CTX**	**CEF**	**CIP**	**TET**	**VAN**
LF	**82**	16	0.25	8	64	32	64	0.25	2
LF	**83**	64	32	>128	>128	>128	64	0.25	1
LF	**84**	32	0.25	16	64	32	128	0.25	1
LF	**85**	32	16	>128	>128	>128	128	0.25	1
LF	**86**	16	0.25	>128	>128	>128	128	0.5	2
LF	**87**	64	0.5	>128	>128	>128	128	0.25	2
LF	**88**	32	0.5	32	128	128	128	0.5	2
LF	**89**	64	0.25	>128	>128	>128	128	0.5	1
LF	**90**	32	0.25	64	128	64	16	0.25	1
LF	**91**	64	32	>128	>128	>128	128	0.25	1
LF	**92**	16	0.25	32	64	32	16	0.25	2
LF	**93**	16	32	>128	>128	>128	128	0.25	1
LF	**94**	16	0.25	32	64	32	128	0.25	1
LF	**95**	8	0.25	4	8	4	128	0.25	2
LF	**96**	64	0.25	16	64	32	128	0.25	1
LF	**97**	32	0.25	32	64	64	16	0.25	2
LF	**98**	32	0.25	128	>128	>128	16	0.25	2
LF	**99**	16	0.5	32	64	32	128	0.25	1
LF	**100**	32	0.25	128	>128	128	128	0.12	0.5
LF	**101**	16	0.25	32	64	32	128	0.25	1
LF	**102**	32	0.25	128	>128	128	>128	0.25	1
LF	**103**	32	0.25	32	128	32	32	0.25	1
LF	**104**	2	0.06	16	16	32	1	0.25	2
LF	**105**	4	0.5	32	64	32	32	0.12	0.5
LF	**106**	32	0.25	16	64	32	128	0.25	1
LF	**107**	32	0.25	32	128	64	128	0.25	1
LF	**108**	32	0.5	32	128	64	128	0.25	1
LF	**109**	64	0.5	>128	>128	>128	128	0.25	1
LF	**110**	16	0.5	32	64	32	8	0.25	1
LF	**111**	32	0.5	32	64	32	32	0.25	1
LF	**112**	32	0.5	32	128	64	128	0.25	1
LF	**113**	32	0.5	32	128	64	64	0.25	1
LF	**114**	32	0.5	32	128	32	32	0.25	1
LF	**115**	32	0.5	32	>128	64	128	0.25	1
LF	**116**	32	0.5	64	128	64	64	0.25	1
LF	**117**	32	0.5	16	64	32	128	0.25	1
LF	**118**	32	0.5	32	128	32	128	0.25	1
LF	**119**	32	0..5	16	32	16	16	0.25	1
LF	**120**	32	0.5	32	128	128	128	0.25	1

AMP: ampicillin, **GEN**: gentamicin, **OXA**: oxacillin, **CTX**: cefotaxime, **CEF**: cefuroxime, **CIP**: ciprofloxacin, **TET**: tetracycline, **VAN**: vancomycin.

These isolates were also examined (by student Lindsay Lai under the supervision of Dr Leila Vali; Vali *et al*, 2008) by PCR for the presence of the staphylococci biocide resistance genes *norA, qacA/B, qacG, qacH* and *smr*, and for the presence of the *blaZ* β-lactamase gene. NorA is a fluoroquinolone efflux pump which pumps out dyes and quaternary ammonium compounds (QACs). The *smr* and *qac* genes encode an energy-

dependant export system. Whilst they mainly confer reduced susceptibility to QACs, *smr*, *qacA/B* and *norA* also confer reduced susceptibility to biguanides, including chlorhexidine. Of these 120 clinical MRSA isolates, 97.5% contained *blaZ*, 44.2% contained *smr*, 36.7% contained *norA*, 8.3% contained *qacA/B* and 3.3% contained *qacH*. All the isolates with *qacA/B* also contained *blaZ*. However, not all of the isolates with *blaZ* contained *qacA/B.*

6.2.3 Selection of Isolates for Further Study

Three isolates were selected for use in the biocide studies because they represented three different susceptibility profiles: LF 26 contained only *blaZ* and was resistant to most tested antibiotics except gentamicin, tetracycline and vancomycin; LF 67 contained *qacH, blaZ* and *smr* and was only resistant to cefotaxime, cefuroxime and tetracycline; and LF 93 contained *norA, blaZ* and *smr* and was resistant to all antibiotics tested except tetracycline and vancomycin (Vali *et al*, 2008).

6.2.4 Controls Testing Neutraliser Toxicity and Efficacy

Comparison of viable counts of the control strain EMRSA-16 with and without the use of a neutralizer (as described in Section 2.11.5) showed that the neutraliser successfully quenched the effect of chlorhexidine at all concentrations examined, with no significant difference observed in the mean counts of quenched chlorhexidine compared to a control with water alone ($P > 0.05$). Similarly, comparison of the counts with neutraliser

compared to water alone showed that the neutraliser had no significant toxic effect upon the bacteria ($P > 0.05$).

Table 6.2 Comparison of mean counts for neutralizer controls

	Mean cfu/ml			T-test *
C	1.43×10^7	3.67×10^6	2.10×10^7	
N	1.23×10^7	1.97×10^6	3.07×10^7	P = 0.66
NT	6.33×10^6	3.00×10^6	2.20×10^7	P = 0.45

C – Control count with water alone.
N – Neutraliser efficacy control – neutraliser applied before CHX.
NT – Neutraliser toxicity control – neutraliser applied instead of water.
* Paired T-test, two-tailed distribution. P>0.05 means no significant difference between means.

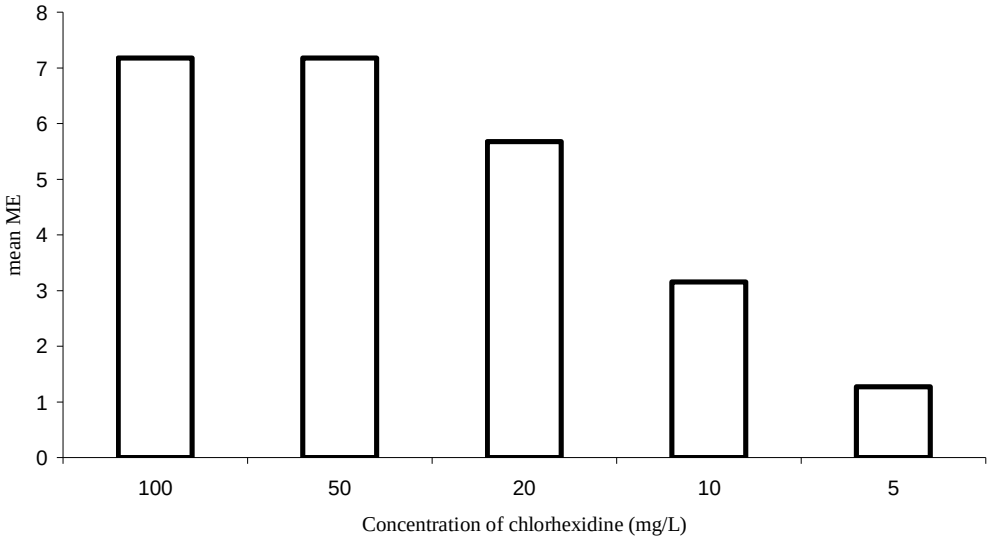
6.2.5 Quantitative Suspension Test

When the efficacy of biocides upon bacteria is being considered, it is the lethal effects rather than the inhibitory effects (as would be measured by MICs) which are more important. Biocides are generally required to kill bacteria and are therefore used in excess of MIC levels, so suspension tests rather than MIC tests are often used as a simple and more effective preliminary evaluation of the antibacterial activity of a biocide (Thomas *et al*, 2005). Microbiocidal Effect (ME) measures the log reduction in cfu/ml after biocide contact, calculated as shown in Section 2.11.4.

Figure 6.1 shows the mean of three results of the ME of 5 to 100 mg/L chlorhexidine solutions upon a washed suspension of the control strain EMRSA-16. Total killing was

observed at 50 and 100 mg/L of chlorhexidine, hence these figures equate to the log cfu/ml value for the control, and the maximum limit of detection for the experiment.

Figure 6.1 Mean Microbiocidal effect (ME) of chlorhexidine concentrations upon control strain EMRSA-16



It is apparent that a reduction of 50% from 100 mg/L to 50 mg/L chlorhexidine did not result in any loss of efficacy of the biocide for this sample in these test conditions and within the limit of detection. The further reductions in concentration did result in a noticeable reduction in the microbiocidal effect of chlorhexidine, with a decrease from a greater than 7-log reduction in numbers to a just over 1-log reduction when a concentration of 5 mg/L chlorhexidine was used.

However, even at the lowest concentration, an inhibitory effect was still exerted by chlorhexidine. Such concentrations could arise in the hospital environment due to the misuse of biocides; for example if they are not removed after cleaning and are allowed to linger on surfaces.

6.2.6 Controls for Surface Disinfection Tests

Two separate controls were used in the surface disinfection tests for calculating the microbiocidal effect of chlorhexidine. The first control did not take into account the effect of drying on the viability of the bacterial cells, the second was specific not only to each isolate, but also to each drying time for the isolate, in an attempt to account for the loss of viability subsequently observed after 2 and 24 hours of bacterial drying.

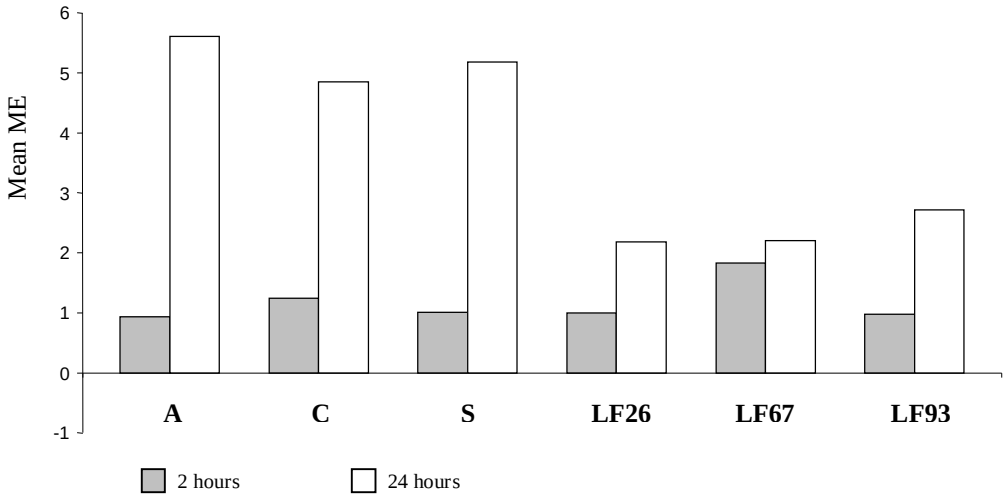
By calculating the non-drying control concurrently with the tests and the drying control, any loss of viability after drying could be observed and accounted for specifically for each isolate.

6.2.7 Surface Disinfection Test

This test aimed to simulate a situation that may arise in the clinical environment, that of low concentrations of chlorhexidine solutions being used to clean up bacterial residue on surfaces (see Sections 1.2 and 6.1). Initial surface disinfection tests were performed and microbiocidal effects calculated from one control for each isolate (taken at time 0, with no drying having taken place), not taking account of any drying effect. Figure 6.2 shows

the subsequently calculated mean microbiocidal effect of 100 mg/L chlorhexidine on each of the tested isolates, after 2 and 24 hour drying times. There were not enough results to establish whether or not there were statistically significant differences between observations, and as such error bars are not included in the following figures, which show the mean of three results.

Figure 6.2 Mean MEs of the isolates after 2 and 24 hour bacterial drying times, calculated compared to a control without drying



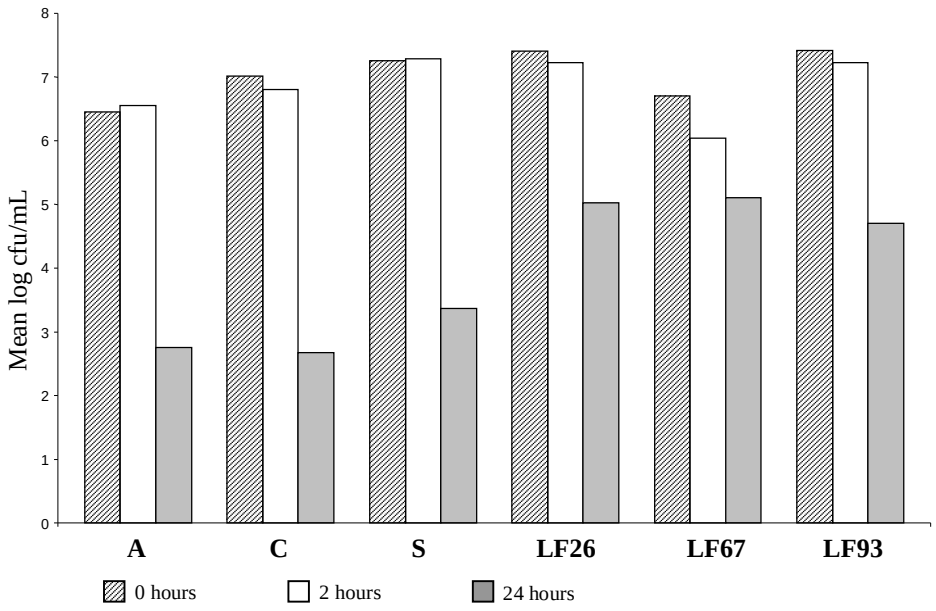
A – *Acinetobacter baumannii* ATCC 19606; **C** – EMRSA–16; **S** – *Staphylococcus aureus* NCTC 6571; **LF 26, 67, 93** – clinical MRSA isolates.

After only 2 hours of drying, the ME of chlorhexidine was less than a 2-log reduction for all tested isolates and control strains. However the effectiveness of chlorhexidine increased in all cases after 24 hours of bacterial drying, to a greater extent in the control strains compared to the clinical isolates.

After 24 hours bacterial drying there was a pronounced difference in the microbiocidal effect of chlorhexidine upon the control strains compared to the clinical isolates; a much greater effect was observed against the control strains than against the clinical isolates.

However, a reduction in the viability of the bacterial isolates was observed following surface drying of the bacterial solutions, as shown in Figure 6.3 which displays the mean (3 results) log cfu/mL for the non-drying control (0 hours) and two drying controls (2 and 24 hours) for each isolate. In most isolates there is clearly some reduction after 2 hours of drying, and a marked reduction in bacterial viability for most isolates after the cells have been surface dried for 24 hours.

Figure 6.3 Mean log cfu/mL of non-drying control (0 hour) and the 2 and 24 hour drying controls for each sample



A – *Acinetobacter baumannii* ATCC 19606; **C** – EMRSA–16; **S** – *Staphylococcus aureus* NCTC 6571; **LF 26, 67, 93** – clinical MRSA isolates.

This difference appeared more pronounced in the control strains than in the clinical isolates, possibly suggesting that the clinical isolates sampled were more resilient to the drying conditions. Taken with the previous results shown in Figure 6.2, this suggests that the reduced microbiocidal effect of chlorhexidine compared to the control strains is in part to the greater resilience of the clinical isolates to the effects of drying.

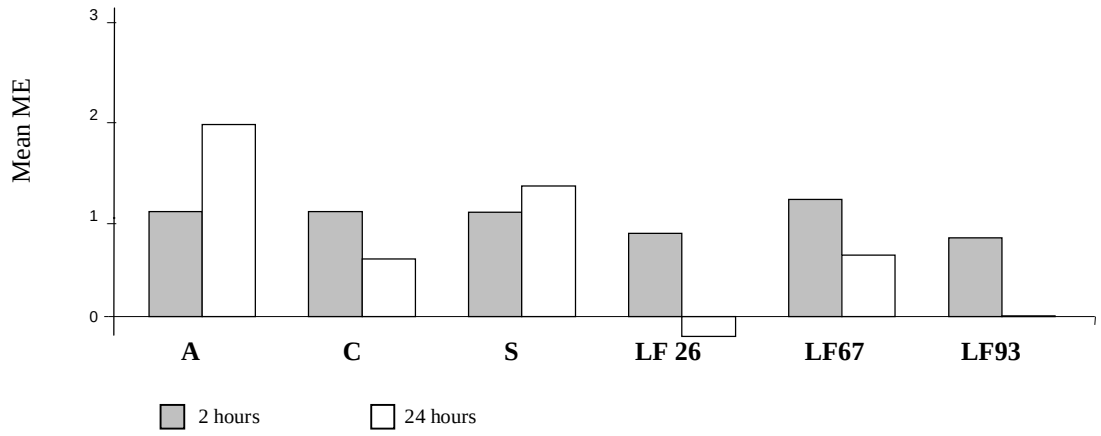
To attempt to account for the impact of surface drying upon the calculated microbiocidal effects of chlorhexidine, further surface disinfection tests were performed using drying controls specific for each isolate and each drying time. Figure 6.4 shows the mean microbiocidal effect of chlorhexidine upon the isolates, as calculated using these drying controls.

It is apparent that the difference in the effect of chlorhexidine upon control strains and the clinical isolates was much less pronounced than that seen in Figure 6.2, where microbiocidal effect was calculated from a non-drying control and the effect of drying on viability was not accounted for. However there was still reduced efficacy of chlorhexidine upon the clinical isolates generally compared to the standard strains, especially after 24 hours bacterial drying for LF26 and LF93.

Of importance aside from any differences between control strains and clinical isolates is that even after 24 hours of bacterial drying chlorhexidine did not appear to exert a greater than 4-log reduction on any of the isolates, once the effect of drying upon bacterial viability was taken into account. This is less than the “equal to or greater than

5-log reduction in numbers of challenged organisms" effect which is suggested as the desired efficacy level for biocides (Payne *et al*, 1999). Additionally, this reduction was much less than the ME at this concentration in the quantitative suspension tests performed upon EMRSA-16.

Figure 6.4 Mean MEs of the isolates after 2 and 24 hour bacterial drying times, calculated compared to 2 and 24 hour drying controls respectively



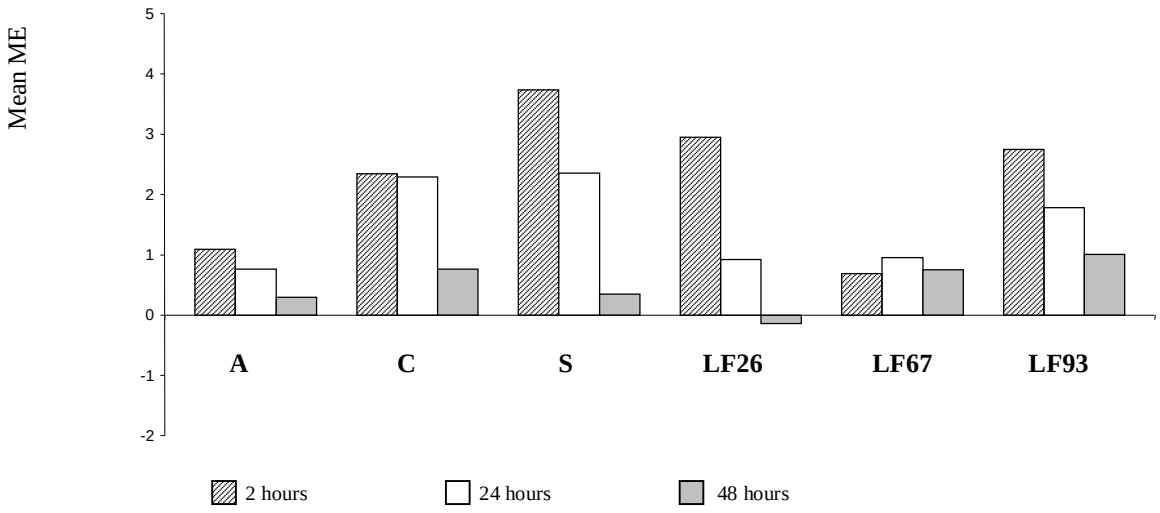
A – *Acinetobacter baumannii* ATCC 19606; **C** – EMRSA–16; **S** – *Staphylococcus aureus* NCTC 6571; **LF 26, 67, 93** – clinical MRSA isolates.

6.2.8 Biocide Residue Test

In this test the effect of biocide residues on the clinical isolates was established. In the clinical environment, it may be that inappropriate use leaves a biocide residue on surfaces and that low levels of chlorhexidine are therefore being used (as mentioned in Sections 1.2 and 6.1). It is therefore important to investigate whether this residue exerts any effect upon the exposed bacteria. Figure 6.5 shows the mean (3 results) ME of 25

mg/L chlorhexidine dried for 2, 24 and 48 hours against the control strains and clinical isolates.

Figure 6.5 Mean MEs of the isolates after 2, 24 and 48 hours biocide drying times



A – *Acinetobacter baumannii* ATCC 19606; **C** – EMRSA–16; **S** – *Staphylococcus aureus* NCTC 6571; **LF 26, 67, 93** – clinical MRSA isolates.

Generally the efficacy of the chlorhexidine residues decreased with longer drying times. Within the control strains, the sensitive *S. aureus* strain seemed more susceptible to chlorhexidine than either EMRSA-16 or the *A. baumannii* strain, as seen by the greater mean ME. Even after 2 hours of biocide drying, there was a much lower ME of chlorhexidine upon the *A. baumannii* strain. There did not appear to be any marked difference between the ME of chlorhexidine on clinical isolates taken as a whole,

compared to control strains. However there was a low ME observed for all residue drying times in LF67.

Importantly, an effect was still being exerted by chlorhexidine after drying, in all cases after 24 hours, and in all but one (the clinical isolate LF 26) even after 48 hours of drying. The effect was minimal, hence not sufficient to kill all bacteria, which may allow the less susceptible bacteria in the population to become selected out.

6.2.9 Post Biocide Residue Exposure MICs

The MICs of the control strains and the isolates were determined following their contact with each of the chlorhexidine residues. Table 6.3 shows the MICs for the originally tested antibiotics after exposure to chlorhexidine residues dried for 2, 24 and 48 hours, compared to the initial MICs where there had been no exposure.

For the majority of isolates there was little or no change in the MICs of the antibiotics tested after exposure to chlorhexidine dried for the different times. However there were substantial increases (greater than one concentration step) observed in the MICs of several antibiotics against EMRSA-16 and clinical isolate LF 26, and of all antibiotics against *S. aureus* strain NCTC 6571, after exposure of the isolates to chlorhexidine residues dried for 48 hours (Table 6.3).

Table 6.3 Post-chlorhexidine exposure MICs (mg/L) for the tested clinical isolates and control strains after various biocide drying times.

	Hours drying	AMP	CTX	VAN	GEN	CIP	CEF	TET	OXA
ATCC	0	128	16	128	2	1	64	2	128
19606	2	>128	16	128	4	2	64	2	128
(A)	24	128	16	128	2	1	64	2	128
	48	128	16	128	2	1	64	2	128
EMRSA	0	>128	8	1	0.5	1	8	2	4
16	2	>128	8	1	0.5	2	8	2	8
(C)	24	>128	8	1	0.5	2	4	2	4
	48	>128	16	**128**	**2**	2	**64**	2	**128**
NCTC	0	0.06	1	1	0.25	0.25	4	0.5	0.12
6571	2	0.06	1	1	0.5	0.25	1	0.25	0.12
(S)	24	0.002	1	0.002	0.25	0.002	0.002	0.002	0.002
	48	**128**	**32**	**>128**	**2**	**2**	**64**	**1**	**128**
LF 26	0	32	64	1	0.25	64	128	0.5	32
Clinical	2	64	128	2	0.25	64	>128	0.5	64
MRSA	24	64	128	2	0.12	64	>128	0.5	64
	48	**128**	128	**4**	0.25	64	>128	0.5	64
LF 67	0	4	4	4	0.016	1	4	64	0.5
Clinical	2	4	4	4	0.016	1	4	64	0.5
MRSA	24	4	4	4	0.016	1	4	64	0.5
	48	4	4	2	0.016	2	4	32	0.5
LF 93	0	32	>128	2	32	128	>128	0.5	>128
Clinical	2	32	>128	2	32	128	>128	0.5	>128
MRSA	24	32	>128	2	32	128	>128	0.5	>128
	48	32	>128	2	64	128	>128	0.5	>128

AMP– ampicillin, **CTX**– cefotaxime, **VAN**- vancomycin, **GEN**– gentamicin, **CEF**– cefuroxime, **TET**– tetracycline, **OXA**– oxacillin.

Most worryingly, this included a change from an MIC of 1 mg/L of vancomycin to 128 and >128 mg/L in EMRSA-16 and NCTC 6571 respectively, and an increase to 4 mg/L vancomycin for the clinical isolate LF 26, approaching the resistance level (breakpoint >8 mg/L). Of particular concern is that the MICs against the *S. aureus* strain for all antibiotics tested had increased, leading to a previously sensitive strain becoming more resistant across the spectrum of antibiotic usage.

6.3 Discussion

The work described in this Chapter formed part of a study into the effects of chlorhexidine, the frequency of biocide resistance genes, and antibiotic resistance of MRSA, and has subsequently been published (Vali *et al*, 2008; Appendix 1). The tests were intended to mimic situations that may arise in the clinical environment such as when chlorhexidine is used incorrectly or when poor infection control practices exist, leading to residues of biocide or bacteria on surfaces, or to non-lethal concentrations of biocide being used.

Whilst there may be debate about the clinical relevance of results testing sub-lethal concentrations of chlorhexidine, as it is intended for high in-use concentrations, research into low concentrations is increasingly being advocated due to their incorporation in many products and the likelihood of low concentrations being present in the clinical environment (Section 1.2.9; Levy, 2001; Bloomfield, 2002; Russell, 2003; Maillard, 2007).

As would be expected, the efficacy of chlorhexidine was reduced when applied to bacterial residues and the ME decreased with longer bacterial drying times. Once the effect of drying upon bacterial viability had been taken into account by the use of controls specific to each drying time, the results from the surface disinfection tests did not demonstrate a clear difference between the efficacy of chlorhexidine against control strains compared to the clinical isolates.

However the results of the counts at each time did suggest that the viability of the *S. aureus* and EMRSA-16 strains may have been more affected by surface drying than the clinical isolates. This would at least partially account for the clear difference in the ME of chlorhexidine upon control strains and clinical isolates shown in Figure 6.2 (where the controls did not take into account the effect of drying).

The observation of variation in the efficacy of chlorhexidine against the clinical isolates tested is disconcerting in itself as it may indicate that certain isolates are able to survive chlorhexidine exposure and be selected out of the population under this selective pressure, a critical concern in the issue of the development of reduced susceptibility to biocides (Sections 1.2.8 & 1.2.9; Bloomfield, 2002; Russell, 2003).

The prevalence of biocide resistance genes in the clinical MRSA population in general and in the three clinical isolates tested in more detail was varied (Vali *et al*, 2008), which is likely to account for the variation in the ability of the clinical isolates to survive the presence of low concentrations of chlorhexidine.

When residues of chlorhexidine were tested for their efficacy against bacterial suspensions, it was clear that even after 48 hours of biocide drying there was an effect being exerted by the biocide upon most isolates, both control and clinical. This was a low level inhibition, and as such is a cause for concern as it supports the concept that chlorhexidine residues in the hospital environment, as might occur through the misuse of

the biocide, can act as a selective pressure allowing less-susceptible bacteria in a population to survive through incomplete eradication (Sections 1.2.8 and 1.2.9; Bloomfield, 2002; Russell, 2003).

The observation of increased MICs of several antibiotics after exposure to chlorhexidine, is of critical importance as it supports the concern that reduced susceptibility in biocides may be correlated to increased resistance in antibiotics, as has been observed in other work (detailed in Sections 1.2.8 and 1.2.9). Increases were not only observed in the control EMRSA-16 strain, but also in a current clinical isolate from the NRIE (LF26) and, of particular concern, the sensitive *S. aureus* control strain demonstrated an increase in all of the tested antibiotics after exposure to chlorhexidine residues that had been drying for 48 hours.

It may be partly that the minimal effect exerted by the chlorhexidine residue allowed a greater number of the population to survive, thereby increasing the chance of survival against each antibiotic, but that resistance was not actually selected by exposure to biocide. Equally, selection for over-expression of efflux pumps with both antibiotic and biocide substrates may be a factor in this observation, as has been reported elsewhere; *E. coli* mutants selected for reduced susceptibility to pine oil also showed resistance to several antibiotics and over-expression of the *marA* gene, which activates the multidrug AcrAB efflux pump (Moken *et al*, 1997; Levy, 2002b).

Similarly, in this study all of the clinical MRSA isolates which contained *qacA/B* also contained *blaZ*, as has previously been reported. However, not all of those with the *blaZ* gene contained *qacA/B*. This suggests that biocide reduced susceptibility mediated by the *qacA/B* gene may be a risk factor for acquisition of antibiotic resistance (mediated by the *blaZ* gene) but that antibiotic resistance may not necessarily encode biocide resistance (Vali *et al*, 2008). It may be that further antibiotic genes are similarly borne on plasmids alongside biocide resistance genes, and that residues of chlorhexidine selected for the biocide resistance genes whilst subsequently co-selecting for antibiotic resistance.

Vancomycin resistant *Staphylococcus aureus* (VRSA) is not yet widespread but is a cause for concern, particularly as VanA-type resistance may result via acquisition from more widespread vancomycin resistant entericocci (VRE) (Foucault *et al*, 2009). Additionally, vancomycin-heteroresistant strains have been reported, which may complicate analysis and surveillance of this problem (Linden, 2008). From the results of the present study, the observed increase in vancomycin MIC to unusually high levels is particularly disturbing, as isolates with such high resistance are unreported in natural clinical populations, and such dramatic increase in resistance following biocide exposure is also unreported. Chlorhexidine and other biocides are already in widespread use in the hospital environment. The implication from these results that chlorhexidine residues may induce this unusual increase in antibiotic resistance, especially in previously sensitive *S. aureus* strains as well as MRSA isolates, threatens biocide usage and also treatment options, and requires urgent further investigation.

Initially the biocide residue test would need to be repeated several times on a range of strains to examine the reproducibility of these observations. It would be useful too to develop a modified technique whereby the biocide and neutraliser are removed from the solution before re-culturing for measurement of the post-exposure MIC, as it may be that they interfere with MIC measurements. Additionally, isolates could be tested before and after biocide exposure for further biocide and antibiotic resistance genes, including *vanA*, and the fitness of post-exposure 'mutants' could be tested to determine the stability of the increased MICs observed. It has recently been suggested that the induction of VanA-type resistance is highly costly for MRSA, but minimal when not induced (Foucault *et al*, 2009). If due to this mechanism, the observed increase in vancomycin MIC may therefore not be stable, but the presence of *vanA* and its ready induction by chlorhexidine exposure, if responsible for the increase, would have serious implications for the potential spread of VRSA clinical isolates.

This study highlights the importance of efficacy testing on clinical isolates and not just on the standard strains which are currently used, as there is clearly variation within the clinical population. This agrees with reports of reduced susceptibility of MRSA strains to chlorhexidine, compared to MSSA strains (Kampf *et al*, 1998). Screening a biocide against a panel of the problem strains within specific hospitals or regions to determine efficacy and highlight any potential problems before it is introduced would be ideal, but logistically this would be difficult to implement.

The results also re-emphasise the need for good infection control practices, and the correct use of chlorhexidine in particular and biocides in general so that they may continue to be useful in the fight against infection; the targeted, rather than excessive, use of biocides has been increasingly advocated, so that they may retain their usefulness in preventing infection where they are most needed (Bloomfield, 2002; Maillard, 2007; Russell, 2003). It may not seem obvious to the general public that residues of a substance designed to eradicate bacteria may in fact encourage the survival of the worst of the bacterial population, but this is an aspect that needs to be brought to the attention of at least any hospital workers responsible for the use of biocides. Of course it may be that the increasing use of biocides such as chlorhexidine in the domiciliary environment also plays, or will play, a part in any development of reduced biocide susceptibility, but unfortunately that was out with the scope of this study.

The potential problem of reduced susceptibility to biocides and the techniques used to investigate this area are a relatively new concern compared to that of antibiotic resistance, and as discussed (Section 1.2) there is lack of internationally approved and reproducible methods to test susceptibility. The methods used here are based on previously described work (Thomas *et al*, 2005), incorporating some aspects of methods used to test the efficacy of biocides (Payne *et al*, 1999). However these techniques proved quite limiting in practice for the analysis of many isolates, as would have been useful to determine the effect of chlorhexidine on clinical isolates within the hospital population. It proved logistically impossible to test more than a few isolates at one time, due to amount of materials needed and the timescale required, and of course repetition

was required to gain more meaningful results. The results also highlight the importance of using proper controls to account for variations such as the effect of drying upon bacterial cells as in the surface disinfection test, demonstrated in Figure 6.2 (without drying controls) and Figure 6.4 (with a drying control).

For future work, and to allow the essential screening of many isolates, it would be useful to develop a technique, possibly utilizing ELISA plates and a plate reader, which would enable the testing of biocide effect upon a large number of isolates at once, giving a greater number of quick results and allowing better control of conditions such as temperature, innoculum concentration and timings, leading to more reproducible results. This could also be a useful technique for the screening of specific hospital isolates before the implementation of the use of a biocide, as mentioned above.

Within the actual hospital environment there are of course other factors which may influence the effects of chlorhexidine such as temperature, other suspensions and fluids, and surface contaminants (Section 1.2; Bloomfield, 2002). Whilst it would be possible to take some account of such factors, it was not within the scope of this study, and may be another aspect to bear in mind for any future work on this subject.

There is increasing reliance upon biocides as a means to control infection by and spread of both HA-MRSA and emerging CA-MRSA in the clinical environment in light of the growing resistance to antibiotics (Section 1.2); as such the development of reduced susceptibility to chlorhexidine and the possible correlation with antibiotic resistance is a

serious cause for concern. Additionally, the rise of CA-MRSA and the increased use of biocides in domiciliary products may in theory promote reduced susceptibility to biocides and associated antibiotic resistance in CA-MRSA and, given the reports of movement of these strains within hospitals, this could also exacerbate any problems arising from HA-MRSA reduced susceptibility to biocides (Sections 1.2.4 & 1.2.6).

As hypothesised (Section 1.6.5), these results indicate that low concentrations of chlorhexidine, as may be present in the clinical environment, could exert selective pressure on clinical MRSA isolates by producing a minimal effect and that this may lead to cross-resistance to antibiotics. Additionally, there are differences in susceptibility to these low concentrations between clinical isolates and compared to standard strains. Most importantly, the increases in antibiotic MICs following exposure to chlorhexidine, particularly the unusual increase observed for vancomycin MICs, may have dramatic implications for biocide use in both the hospital and domiciliary environments and requires urgent attention. Biocides are a fundamental tool in the fight against hospital infections, but they will only remain such when used properly; as these results suggest, it may be that incorrect use could dramatically worsen the situation.

Chapter 7: Conclusions

This study has aimed to examine whether there is cause for concern regarding reduced biocide susceptibility in MRSA, and to further understanding of what factors may set certain *Acinetobacter* spp apart from others in their ability to acquire resistance. Rapid resistance development is likely to arise via a complex interplay of factors, both environmental and bacterial; therefore, examining specific aspects in isolation has limitations. However, whilst it can be difficult to link laboratory studies with the clinical situation, the benefit of such investigations is indisputable, especially for predicting worse case scenarios and examining the potential of new threats.

7.1 *Acinetobacter* spp

Regarding the hypothesis (Section 1.6.2) that distinct sub-populations of *Acinetobacter* spp clinical isolates exist, with varied *mutS* genes and an increased ability to develop antibiotic resistance, the results presented here show that this could be possible. The aims (Section 1.6.3) were to speciate clinical *Acinetobacter* spp isolates of varying antibiotic sensitivities, characterise and compare their *mutS* gene to non-clinical isolates and within the clinical population in order to establish any correlation between susceptibility and *mutS* type, and to assess whether any differences in *mutS* were associated with varying ability to develop ciprofloxacin resistance.

Acinetobacter spp clinical isolates with varying susceptibilities were speciated and their *mutS* gene sequenced, allowing comparison. There was variation in the *mutS* gene, compared to non-clinical strains and between clinical isolates, which was associated with different antibiotic susceptibilities. A novel *mutS* amino acid sequence (the R-type *mutS* sequence) was discovered, highly conserved in clinical multi-resistant, resistant and outbreak *Acinetobacter* isolates. Furthermore, mutation studies found that possession of this *mutS* sequence in intermediate *Acinetobacter* spp clinical isolates was associated with increased ability to develop clinically significant ciprofloxacin resistance in response to challenge.

An increased mutation frequency itself did not correlate well with either possession of the R-type *mutS* gene, nor the ability to develop significant ciprofloxacin resistance, which agrees with the caution concerning the use of an increased rate of mutation alone to imply an increased ability to develop resistance (Section 1.5.5; Martinez & Baquero, 2000). It may be that fitness costs in some isolates were not successfully ameliorated by the parallel development of compensatory mutations (Section 1.5.3). Increased transformation frequencies due to deficient mismatch repair, as has been reported in *mutS* defective *Salmonella* spp and in *A. baylyi* strain ADP1 (Section 1.5.4), may contribute to resistance development in this situation; the ability to take on exogenous resistance genes from a greater reservoir of bacteria in the hospital environment may lead to a more rapid development of resistance. Furthermore, the growing reports of *Acinetobacter* spp associated with infections out with the hospital environment (Section

1.3.3; Anstey *et al*, 2002; Davis *et al*, 2005) may mean there is an even greater reservoir of resistance genes available.

Variation via mutation is fundamental to the development of antibiotic resistance. However the success of clonal strains of multi-resistant bacteria suggests that an effective combination of resistance factors is maintained in bacterial populations; hence it is likely that the conserved R-type *mutS* sequence present in resistant and outbreak *Acinetobacter* spp (Chapter 4) is of importance in the success of these isolates. This ever-increasing similarity with increasing resistance is also indicated by the reduced range of ciprofloxacin MICs observed in developing mutants, and the lack of other mutations observed in the *gyrA* gene of mutants that developed target site mutations (Chapter 5). It may be that the *mutS* gene variation observed here in the sensitive and intermediate *Acinetobacter* spp clinical isolates is indicative of their position in the population as emerging-resistant isolates, progressing from the non-clinical strains to the resistant clinical isolates, and as such they may be progenitors of the resistant clinical strains. Phylogenetic studies would be needed to expand upon this theory.

The correlation of *mutS* type with increased ability to develop resistance is not evident solely within the *A. baumannii* species; the potential importance of gen.sp. 13TU in particular was highlighted, confirming increasing reports that this is also an important clinical species of the *Acinetobacter* genus (Section 1.3.1; Spence *et al*, 2002; Dijkshoorn *et al*, 2007). Additionally, other species may also have relevance in the clinical environment; indeed isolates of other *Acinetobacter* spp were able to develop

mutants with high ciprofloxacin MICs (Chapter 5), suggesting that they may also have potential, under certain conditions, to develop significant resistance.

The grouping of gen.sp. 3 with *A. baumannii* and gen.sp. 13TU as the Acb complex is questionable in light of these results; gen.sp. 3 isolates did not possess the R-type *mutS* sequence and moreover did not seem capable of developing significant resistance. This agrees with cautions from others about this grouping of distinct species (Section 1.3.2; Dijkshoorn *et al*, 2007) and highlights the importance of speciation of isolates belonging to the *Acinetobacter* genus.

Despite the association between *mutS* gene variation and an increased ability to develop resistance, direct cause and effect cannot be demonstrated by these results alone, though they do suggest that this area merits further investigation. If differences in the *mutS* gene are a factor in causing certain *Acinetobacter* isolates to predominate in the clinical environment, this could have implications for the treatment, control and surveillance of these isolates. For example, analysis of the *mutS* gene could be used to determine which emerging-resistance isolates are likely to progress to multi-resistance, a 'hit them hard' approach might be advocated for treatment of infections likely to contain mutator cells, as advocated for treatment of *P. aeruginosa* (Kenna *et al*, 2007), and it may be that research into future treatment options could focus on this aspect of *Acinetobacter* resistance development. Additionally, the increasing importance of other species of *Acinetobacter* in the clinical environment is an area that must be addressed with thorough surveillance and multi-centre analysis; if species other than *A. baumannii* are

of growing concern both as infectious agents and as a potential reservoir of resistance genes for the more prevalent isolates, then surveillance must also include these species.

Further investigations should focus on establishing whether there is a causal link between *mutS* variations and resistance development; rescue of defective *mutS* alleles by recombination has been demonstrated in *E. coli* (Brown *et al*, 2001) and a similar method could enable determination of whether it is, in fact, the *mutS* type that is responsible for the observed increase in ability to develop resistance. It would also be of benefit to examine a wider range of *Acinetobacter* spp to see whether the observations of this study hold true for a greater sample; likewise a wider range of resistance mechanisms could also be examined. Additionally the aforementioned phylogenetic studies would be useful to discover whether the distinct isolates now current have derived from common progenitors, and population dynamics could be investigated by competition studies of isolates with different *mutS* genes to see which proliferated, both with and without antibiotic challenge.

7.2 MRSA

Regarding the hypothesis (Section 1.6.5) that exposure to low rather than the recommended in-use concentrations of biocide in the clinical environment may encourage reduced susceptibility and cross-resistance to antibiotics in MRSA, the results presented here (Chapter 6) show that this may indeed be a possibility. The aims (Section 1.6.6) were to assess the efficacy of low concentrations of chlorhexidine to determine

whether there was variation between clinical MRSA isolates and variation compared to standard strains, and to determine whether exposure to chlorhexidine was associated with increased antibiotic resistance in clinical MRSA isolates.

The efficacy of low concentrations of chlorhexidine was tested upon clinical MRSA isolates and variation was found between them. Additionally, there was variation in the susceptibility of clinical isolates to chlorhexidine compared to standard strains. Furthermore, increases in antibiotic MICs were found after exposure of clinical MRSA isolates to chlorhexidine residues. Whilst biocides are designed for use at high, lethal concentrations, misuse could easily lead to residues or diluted product and hence the presence of low concentrations in the clinical environment. It has been thought that, at low concentrations, biocides act more as antibiotics, exerting non-lethal effects and thereby allowing isolates with reduced susceptibility to survive their presence and have an advantage if challenged again (Section 1.2.9; Bloomfield, 2002). The results presented here (Chapter 6) show that even low concentrations and residues of chlorhexidine exert a minimal effect upon clinical MRSA isolates, thereby providing selective pressure.

Additionally, although further investigation would be required to confirm the clinical relevance, the increase in a range of antibiotic MICs after exposure to chlorhexidine residue, particularly the dramatic increase in vancomycin resistance, is a great cause for concern, and adds weight to previous reports of the possibility of cross-resistance between reduced susceptibility to biocides and resistance to antibiotics (Section 1.2.9;

Moken *et al*, 1997; Levy 2002b; Kõljalg *et al*, 2002). This could have dramatic consequences for the management of infections, colonised patients, and the use of biocides in the hospital environment. The varying efficacy of chlorhexidine upon clinical isolates compared to standard strains suggests that efficacy testing of biocides should be performed on a range of strains including current clinical isolates, to ensure that they are effective against the bacteria which they are aimed against.

The concern regarding reduced susceptibility to biocides is not a new area, but is of growing interest at present with the increased use of and reliance upon biocides in the clinical environment. Whereas there has been much attention directed at antibiotic resistance the same is not true of investigation into biocides and the implication of their greatly increased use, not just in the clinical environment but increasingly in healthy households (Section 1.2.8; Levy 2001; Cookson 2005). The emergence of CA-MRSA, and especially its recent manifestation and movement within the hospital environment, is also disconcerting in light of the rise of biocide-containing products in the home, particularly their prevalence at low levels; in theory this could lead to reduced biocide susceptibility occurring in CA-MRSA, allowing cross-resistance to antibiotics to develop if these strains move into the hospital environment.

Of course, further investigation is needed both to clarify the clinical relevance of the results presented here, and determine whether biocide use in the home is a cause for concern. However, these results highlight areas of concern associated with biocide use and indicate that problems could indeed arise. Given the ineffectiveness of MICs for

testing biocide susceptibility, the development of reliable and internationally agreed methods for quick biocide susceptibility determination of a large number of isolates is important. The growing awareness of the potential impact of reduced susceptibility to biocides has led to calls for surveillance and monitoring of the problem in the clinical environment (Maillard 2007), and such calls seem to be supported by these results. Equally, the use of biocides in a targeted and informed way has been advocated (Bloomfield 2002; Russell 2003); again in light of these results this seems appropriate to prevent overuse and misuse which could lead to the presence of low concentrations.

7.3 General Conclusions

The predominant Gram-positive and Gram-negative nosocomial pathogens, MRSA and *Acinetobacter* spp, are both characterised by multi-drug resistance and emerging problems which jeopardise the ability to control spread and treat infections of these bacteria; control of MRSA is threatened by the potential development of reduced susceptibility to biocides, and the treatment of infections caused by *Acinetobacter* spp is threatened by emerging pan-drug resistance. The work presented here shows that reduced biocide susceptibility could indeed be a threat to the future ability to control MRSA and requires further research, and that the *mutS* gene may play a role in the ability of certain *Acinetobacter* spp to develop clinically significant multi-drug resistance, and likewise warrants further investigation. Whilst this work alone is not enough to win the war against bacteria, it is the adage ‘know your enemy’ that is being addressed.

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Frequency of biocide resistance genes, antibiotic resistance and the effect of chlorhexidine exposure on clinical methicillin-resistant *Staphylococcus aureus* isolates

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***Objectives*: To detect genes conferring resistance to biguanides, quaternary ammonium compounds, β-lactams and fluoroquinolones in clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and to demonstrate whether reduced susceptibility is spread clonally and if the presence of any of the detected genes links to a specific epidemic MRSA. Finally, to identify if exposure to chlorhexidine may cause reduced susceptibility to antibiotics and chlorhexidine.**

***Methods*: In total, 120 clinical MRSA isolates were isolated. *qacA/B*, *qacG*, *qacH*, *norA*, *smr* and *blaZ* genes were amplified by PCR. MICs of eight antibiotics were determined and PFGE was used for typing. Surface disinfection and residue tests were performed for chlorhexidine and a selection of isolates.**

***Results*: *qacA/B* (8.3%), *qacH* (3.3%), *norA* (36.7%), *smr* (44.2%) and *blaZ* (97.5%) were prevalent within the population but *qacG* was not detected. EMRSA-15 (19.2%), EMRSA-16 (15%), P3 (15%) and H (12.5%) were the most common PFGE types. Clinical isolates demonstrated various degrees of susceptibility to chlorhexidine in the surface disinfection [mean microbiocidal effect (ME) = 0–1.91] and biocide residue (mean ME = 0.29–3.74) tests. Increases in post-exposure MICs were observed in both EMRSA-16 and the susceptible *S. aureus* control.**

***Conclusions*: In our study, isolates resembling PFGE type EMRSA-16 harboured more biocide resistance genes than other types. The observed reduction in susceptibility of clinical isolates to chlorhexidine may mean that a selective pressure is being exerted by residues in the clinical environment, and highlights the importance of efficacy testing on clinical strains and good infection control practices. The development of reduced microbial susceptibility to biocides represents a serious cause for concern in the clinical environment.**

Keywords: *qacA/B*, *smr*, *norA*, *blaZ*, PFGE, MRSA

Introduction

Staphylococcus aureus is one of the most important pathogens that causes hospital-acquired infections.[1,2] These infections include soft tissue abscess, endocarditis and bacteraemia. The fact that *S. aureus* is able to exhibit antibiotic resistance means that the clinical environment within a hospital may actively select resistant clones.[2,3] EMRSA-15 and EMRSA-16 (where MRSA stands for methicillin-resistant *S. aureus*) are the two main clones that dominate the UK hospitals.[4] In clinical practice, decontamination and disinfection are the most important intervention measures to prevent bacterial spread. Over the years, the use of biocides has increased[5] including chlorhexidine, probably the most widely used biocide, not only in hand washing and oral products but also as a disinfectant and preservative.[6]

The effectiveness of chlorhexidine in preventing growth of bacterial pathogens may vary with different organisms.[7,8] The efficacy of biocides against standard culture collection strains of *S. aureus*, *Enterococcus hirae*, *Escherichia coli* and

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Pseudomonas aeruginosa is tested as standard and the products that are considered acceptable are those that achieve equal to or greater than a 5 log reduction (99.999%) in numbers of the challenged organisms after 5 min of contact. However, Payne *et al.*[9] have shown that the margin of pass was higher against laboratory culture collection than clinical strains when a short contact time of 1 min was used, and indeed in some clinical strains, resistance to chlorhexidine may develop.[10–12]

One of the resistance mechanisms to antiseptic and disinfectants in *S. aureus* is mediated by an energy-dependent export system encoded by two gene families on the basis of DNA homology and phenotypic properties; *qac* and *smr* (staphylococcal multidrug resistance, also known as *qacC/D*).[13–15] *smr* encodes a protein that functions as a drug pump by an electrogenic drug/proton antiport[15,16] and is usually harboured on small plasmids (<3 kb).[10] *qacA* and *B* genes are closely related and differ at the nucleotide level by seven nucleotides (codon 323). They are usually harboured by large plasmids (>20 kb).[14]

Concomitant antibiotic and biocide resistance have been previously reported in both Gram-negative and Gram-positive bacteria.[5,17] The *blaZ* β-lactamase gene is one of the mechanisms that confers resistance to β-lactam antibiotics in staphylococci. *qacA/B* and *blaZ* usually reside on common plasmids.[15] Also, in staphylococci, the *norA* gene located on the chromosome encodes the fluoroquinolone efflux protein NorA. In addition to fluoroquinolones, it pumps out dyes and quaternary ammonium compounds (QACs) from the bacterial cells to the outer medium.[18]

At least 12 biocide resistance genes have been identified in staphylococci: *qacA-qacJ*, *smr* and *norA*. Although the *qac* genes mainly confer resistance to QACs, *qacA*, *qacB*, *smr* and *norA* confer resistance not only to cationic antiseptics (QACs) but also to biguanides.[19,20]

The aims of this study were to detect *qacA/B*, *qacG*, *qacH*, *norA*, *smr* and *blaZ* genes in clinical MRSA and examine if the presence of any of these genes is linked to a specific epidemic MRSA, PFGE type or antibiotic resistance pattern, and also to identify if exposure to chlorhexidine may cause reduced susceptibility to disinfectants, particularly chlorhexidine, and antibiotics.

Materials and methods

Isolates

One hundred and twenty clinical MRSA were collected from the clinical laboratories of the New Royal Infirmary Edinburgh (NRIE) from February to April 2006 (Table 1).

Table 1. Origin of the clinical MRSA isolates

Origin of MRSA isolates	No. of isolates
Astley Ainslie Hospital	6
Costorphine Hospital	1
Edenhall	1
Liberton Hospital	6
Royal Hospital for Sick Children	1
NRIE	63
Roodlands Hospice	1
Royal Victoria Hospital	4
Western General Hospital	37

MICs

MICs of a panel of antibiotics including ampicillin (breakpoint >2 mg/L), tetracycline (breakpoint >1 mg/L), vancomycin (breakpoint >8 mg/L), gentamicin (breakpoint >1 mg/L), oxacillin (breakpoint >2 mg/L), cefotaxime (breakpoint >4 mg/L), cefuroxime (breakpoint >4 mg/L) and ciprofloxacin (breakpoint >1 mg/L) were determined by the agar dilution method. Bacterial strains were grown overnight and diluted to 10^4 cfu/mL. They were inoculated on plates containing different concentrations of antibiotics (0.016–128 mg/L) with a Denley multipoint inoculator (Denley, Billinghurst, UK) to give a final concentration of 2×10^2 cfu per spot. After inoculation, the plates were incubated overnight. The results were interpreted according to recommendations of the BSAC guidelines.[21] The susceptible *S. aureus* standard strain NCTC 6571, an EMRSA-16 strain and the *Acinetobacter baumannii* standard strain ATCC 19606 were added for comparison.

MICs are inadequate when antiseptics and disinfectants are being considered for the evaluation of antibacterial activity of a biocide.[8,10]

Polymerase chain reaction

PCR was performed with HotStar *Taq* polymerase (Qiagen) according to the manufacturer's instructions and specific primers[15,18,20] for *mecA*, *norA*, *qacA/B*, *qacG*, *qacH*, *smr* and *blaZ* genes are as follows: *mecA*: MecA1, 5′-GTAGAAATGACTGAACGTCCGATA-3′, MecA2, 5′-CCAATTCCACATTGTTTCGGTCTAA-3′; *norA*+2a: 5′-GTAATACCAGTCTTGCCTGT-3′ and *norA*-5: 5′-GTAATGGC TGGTCGTATCAT-3′; *qacG*-F, 5′-CAACAGAAATAATCGGAAC T-3′ and *qacG*-R, 5′-TACATTTAAGAGCACTACA-3′; *qacH*-F, 5′-ATAGTCAGTGAAGTAATAG-3′ and *qacH*-R, 5′-AGTGTGAT GATCCGAATGT-3′; *qacH*-F, 5′-CTTATATTTAGTAATAGCG-3′ and *qacH*-R, 5′-GATCCAAAAACGTTAAGA-3′; *blaZ*-F, 5′-TACA ACTGTAATATCGGAGGG-3′ and *blaZ*-R, 5′-AGGAGAATAAGC AACTATATCATC-3′; *smr*-F, 5′-ATA-AGT-ACT-GAA-GTT-ATT-GGA-AGT-3′ and *smr*-R, 5′-TTC-CGA-AAA-TGT-TTA-ACG-AAA-CTA-3′; and *qacA/B*F, 5′-GCTGCATTTATGACAATGTT TG-3′ and *qacA/B*R, 5′-AATCCCACCTACTAAAGCAG-3′.

PCR products were sequenced by the School of Biological Sciences Sequencing service (SBCSSS), University of Edinburgh.

Pulsed-field gel electrophoresis

Clinical isolates were typed by PFGE with the CHEF-DR II electrophoresis cell after digestion of the cell lysates with *Sma*I restriction endonuclease enzyme.[22] The running parameters were as follows: initial pulse 5 s, final pulse 40 s, at 6 V/cm for 20 h at 14°C. The gels were stained with ethidium bromide and scanned. The analysis of the gels was performed using BioNumerics software version 4.0 (Applied Maths, Ghent, Belgium). This software facilitates the development of the algorithms necessary for the comparison of profiles of isolates, based on the Dice coefficient and the hierarchic unweighted pair arithmetic average algorithm. Cluster analysis and phylogenetic trees were subsequently prepared. The numbering of MRSA was based on EMRSA-15 and EMRSA-16. The rest of the isolates were alphabetically typed.

Solutions used in the tests

Chlorhexidine diacetate hydrate salt (Sigma) was dissolved in sterile distilled water using magnetic stirrers and heat, and filter sterilized through a 0.22 μm filter before use. A stock solution was made and then diluted to the necessary concentrations for each test. The neutralizer solution comprised 0.75% (w/v) azolectin and 5% (v/v) Tween 80 dissolved in sterile distilled water and was autoclave sterilized before use.

Controls

Controls testing the effectiveness and toxicity of the neutralizer were performed for each new batch made against the concentrations of chlorhexidine used.[8] Neutralizer toxicity was evaluated as follows: 1 mL of neutralizer was added to bacteria and left in contact for 5 min. Cells were resuspended, serially diluted and counted using the drop counting method. The number of survivors was compared with those for a control with sterile distilled water replacing the neutralizer, any difference giving an indication of the toxicity, if any, of the neutralizer. The effectiveness of the neutralizer was tested, to ensure the biocide was being quenched as desired, by addition to a mixture of bacteria and biocide, and counting after 5 min of contact time. The count was compared with a sample without biocide quenching, with sterile distilled water replacing the neutralizer. Significance of the difference between controls with water and tests of the effectiveness and toxicity of the neutralizer was determined using the *t*-test to establish *P* values.

Surface disinfection tests

Surface disinfection tests were performed to test the efficacy of chlorhexidine upon surface dried bacterial cultures.[8] In addition to the controls detailed above, a control was performed for each sample to enable the calculation of microbiocidal effect (ME) while taking into account the effect of drying upon the cells. Washed overnight culture (10 μL; 10^8–10^9 cfu/mL) was added to the bottom of a flat-bottomed glass bottle, left to dry for 2 and 24 h and the dried cells were resuspended in 1 mL of sterile water. The mixture was serially diluted and counted using the drop counting method. By comparing counts after the addition of biocide, this control allows for calculation of the log reduction in cell number or ME after exposure to biocide as follows:

ME = number of cfu/mL of the control (biocide and neutralizer replaced by water) − number of cfu/mL after action of the biocide.

For testing the effect of chlorhexidine upon dried bacteria, 10 μL of washed culture was added to the bottom of a 28 mL flat-bottomed glass bottle and left to dry in a laminar air flow cabinet at room temperature for 2 and 24 h. After drying, 0.1 mL of 100 mg/L chlorhexidine was placed over the top of the dried cells and left in contact for 5 min. Neutralizer solution (0.9 mL) was added to stop the reaction and a sterile magnetic stirrer was used to resuspend the cells for 5 min. The neutralized cells were vortexed and serially diluted in sterile distilled water. The number of cells remaining was determined by the drop counting method.

Biocide residue test

This time the effect of biocide residues on MRSA was determined.[8] The control experiments were performed as detailed above. Stock solutions of biocide were prepared in distilled water to give final concentrations of 2.5, 5, 10, 20 and 40 mg/mL. One millilitre of each solution was dispensed into the bottom of a flat-bottomed glass bottle, the excess removed and the bottles left to dry at room temperature for 1, 2, 4, 10, 24, 34 and 48 h. After drying, 20 μL of an overnight culture (10^8–10^9 cfu/mL) or 10^6–10^7 cfu was added to the bottles containing dried biocide residue. The cells were left in contact with the biocide residue for 5 min at room temperature. Neutralizer (1 mL) was added to the mixture and an aliquot was serially diluted in sterile distilled water and counted by the drop counting method.

Again, the ME was calculated by comparison with a control, where water replaced neutralizer and there was no interaction with biocide residue. The MICs for the exposed cells were also determined against the panel of antibiotics to examine the antibiotic susceptibility profiles after exposure of the cells to dried biocide residue. Aliquots of 10 μL of each of these mixtures were inoculated in 10 mL of nutrient broth and incubated at 37°C overnight. MICs were determined as described before.

Statistical analysis

Mean MEs for the surface disinfectant and biocide residue tests were calculated from three results, and *P* values were calculated using the *t*-test to determine whether differences between ME of chlorhexidine at varying time points and for different isolates were significant.

Results

Minimum inhibitory concentrations

Table 2 shows the MICs for the isolates of the antibiotics tested. Of the isolates, 99.2% were resistant to ampicillin, 27.5% to gentamicin, 89.2% to oxacillin, 98.3% to cefotaxime, 90% to cefuroxime, 96.7% to ciprofloxacin and 3.3% to tetracycline. All isolates were susceptible to vancomycin.

Detection of resistance genes

All isolates were *mecA*-positive. *qacA/B* was identified in 10 isolates (8.3%), *qacH* in 4 (3.3%), *norA* in 44 (36.7%), *smr* in 53 (44.2%) and *blaZ* in 117 (97.5%). However, *qacG* was not detected (Table 2). *qacA/B* and *smr* were detected concomitantly in 4.2% (5/120) of the isolates.

PFGE analysis

Figure 1 shows there were three distinct clones consisting of 26 PFGE types. The most common PFGE types were EMRSA-15 (19.2%), EMRSA-16 (15%), P3 (15%) and H (12.5%), a close derivative of EMRSA-16.

Three isolates were selected for further analysis. LF26 with PFGE pattern I, at 3.3% prevalence, contained only *blaZ* and was resistant to most antibiotics tested except gentamicin, tetracycline and vancomycin. LF67, PFGE pattern A at 0.8% prevalence, contained the *qacH*, *blaZ* and *smr* genes and it was only resistant to cefotaxime, cefuroxime and tetracycline. LF93 (PFGE type EMRSA-16) at 15% prevalence was positive for *norA*, *blaZ* and *smr* and was resistant to all antibiotics tested excluding tetracycline and vancomycin.

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Table 2. MICs of antibiotics for clinical MRSA isolated from Edinburgh and the antiseptic resistance genes

	MIC (mg/L)								Antiseptic resistance genes						
Isolate	AMP	GEN	OXA	CTX	CEF	CIP	TET	VAN	*norA*	*qacG*	*qacH*	*blaZ*	*smr*	*qacA/B*	PFGE type
LF101	16	0.25	32	64	32	128	0.25	1	−	−	−	+	−	−	15
LF102	32	0.25	128	>128	128	>128	0.25	1	−	−	−	+	+	−	15
LF107	32	0.25	32	128	64	128	0.25	1	−	−	−	+	−	−	15
LF111	32	0.5	32	64	32	32	0.25	1	−	−	−	+	−	−	15
LF119	32	0.5	16	32	16	16	0.25	1	−	−	−	+	−	−	15
LF29	32	0.12	16	64	128	16	0.25	0.5	−	−	−	+	−	−	15
LF45	32	0.5	64	128	>128	32	0.25	0.5	−	−	−	+	−	+	15
LF71	16	0.5	32	64	64	16	0.25	2	−	−	−	+	+	−	15
LF73	16	0.25	16	16	32	16	0.25	1	−	−	−	+	−	−	15
LF75	16	0.25	32	128	64	64	0.5	2	−	−	−	+	+	−	15
LF78	32	1	64	128	>128	64	0.25	2	−	−	−	+	+	−	15
LF84	32	0.25	16	64	32	128	0.25	1	−	−	−	+	−	−	15
LF92	16	0.25	32	64	32	16	0.25	2	−	−	−	+	−	−	15
LF95	8	0.25	4	8	4	128	0.25	2	−	−	−	+	+	−	15
LF98	32	0.25	128	>128	>128	16	0.25	2	−	−	−	+	−	−	15
LF117	32	0.5	16	64	32	128	0.25	1	+	−	−	+	+	−	15
LF15	16	0.25	8	16	64	128	0.25	0.5	+	−	−	+	−	−	15
LF20	32	0.25	16	64	128	128	0.25	0.5	+	−	−	+	−	−	15
LF22	16	0.25	16	64	64	128	0.25	1	+	−	−	+	−	−	15
LF25	32	0.12	32	32	128	32	0.25	0.5	+	−	−	+	−	−	15
LF28	32	0.12	32	64	>128	64	0.25	0.5	+	−	−	+	−	−	15
LF79	16	0.5	32	128	>128	>128	0.25	1	+	−	−	+	−	−	15
LF81	64	32	>128	>128	>128	128	0.5	2	+	−	−	+	+	−	15
LF109	64	0.5	>128	>128	>128	128	0.25	1	−	−	−	+	+	−	16
LF41	16	32	128	>128	>128	64	0.25	0.5	−	−	−	+	+	+	16
LF47	16	32	32	>128	>128	64	0.25	0.5	−	−	−	+	+	+	16
LF53	64	0.12	0.12	>128	0.25	0.12	0.12	0.5	−	−	−	+	−	−	16
LF59	0.032	0.12	0.12	2	0.12	64	0.12	0.12	−	−	−	+	+	−	16
LF63	16	32	128	>128	>128	128	0.25	1	−	−	−	+	+	−	16
LF11	32	0.25	>128	>128	>128	64	0.25	0.5	+	−	−	+	−	−	16
LF16	16	64	>128	>128	>128	64	0.25	0.5	+	−	−	+	+	−	16
LF34	16	32	128	>128	>128	64	0.25	0.5	+	−	−	+	+	−	16
LF4	32	32	>128	>128	>128	64	0.25	0.5	+	−	−	+	+	−	16
LF5	16	32	>128	>128	>128	128	32	0.5	+	−	−	+	+	−	16
LF74	16	32	128	>128	>128	128	32	1	+	−	−	+	+	−	16
LF76	16	32	>128	>128	>128	128	0.5	2	+	−	−	+	+	−	16
LF80	128	0.5	>128	>128	>128	128	0.5	2	+	−	+	+	+	−	16
LF86	16	0.25	>128	>128	>128	128	0.5	2	+	−	−	+	+	−	16
LF89	64	0.25	>128	>128	>128	128	0.5	1	+	−	+	+	+	−	16
LF9	64	0.5	128	>128	>128	64	0.25	0.5	+	−	−	+	−	−	16
LF93	16	32	>128	>128	>128	128	0.25	1	+	−	−	+	+	−	16
LF67	1	0.25	0.5	4	8	1	32	2	−	−	+	+	+	−	A
LF23	32	0.5	64	128	>128	16	0.25	1	+	−	−	+	−	−	B
LF65	16	0.5	1	8	8	0.5	0.5	2	+	−	−	+	+	−	C
LF105	4	0.5	32	64	32	32	0.12	0.5	−	−	−	+	−	+	D
LF44	64	0.12	64	32	>128	64	0.25	0.5	−	−	−	+	−	+	D
LF58	32	0.12	0.12	32	0.25	64	0.25	0.5	−	−	−	+	−	−	D
LF64	32	0.25	32	16	64	128	0.5	1	−	−	−	+	−	−	D
LF88	32	0.5	32	128	128	128	0.5	2	−	−	−	+	+	−	D
LF70	32	0.25	32	128	128	128	0.5	2	+	−	−	+	+	−	D
LF94	16	0.25	32	64	32	128	0.25	1	−	−	−	+	+	−	E
LF36	16	0.25	8	32	16	64	0.25	0.5	+	−	−	+	−	−	E1
LF104	2	0.06	16	16	32	1	0.25	2	−	−	+	+	+	−	F

Continued

Table 2. *Continued*

Isolate	MIC (mg/L)								Antiseptic resistance genes						PFGE type
	AMP	GEN	OXA	CTX	CEF	CIP	TET	VAN	*norA*	*qacG*	*qacH*	*blaZ*	*smr*	*qacA/B*	
LF39	32	0.12	16	32	32	64	0.25	0.5	+	−	−	+	−	−	G
LF32	32	32	32	32	128	32	0.25	0.5	−	−	−	+	+	−	G1
LF33	32	16	32	32	128	64	0.25	0.5	−	−	−	+	+	−	G1
LF55	32	32	32	32	128	64	0.25	0.5	−	−	−	+	−	−	G2
LF8	32	0.25	64	128	>128	32	0.25	1	−	−	−	+	−	−	G2
LF82	16	0.25	8	64	32	64	0.25	2	−	−	−	+	−	−	G2
LF10	32	0.25	64	>128	>128	128	0.25	0.5	+	−	−	+	−	−	G2
LF3	32	32	64	128	>128	64	0.25	0.5	+	−	−	+	−	−	G2
LF31	64	16	128	>128	1	32	0.12	0.5	−	−	−	+	+	−	H
LF38	64	32	128	>128	>128	64	0.25	0.5	−	−	−	+	+	−	H
LF43	64	16	128	>128	>128	32	0.25	0.5	−	−	−	+	+	+	H
LF54	64	0.12	0.12	>128	0.25	64	0.12	0.25	−	−	−	+	+	−	H
LF57	64	0.12	0.12	>128	0.25	64	0.25	0.5	−	−	−	+	+	−	H
LF100	32	0.25	128	>128	128	128	0.12	0.5	+	−	−	+	−	−	H
LF12	64	8	>128	>128	>128	128	0.25	0.5	+	−	−	+	−	−	H
LF17	64	32	>128	>128	>128	64	0.25	1	+	−	−	+	−	−	H
LF18	64	64	>128	>128	>128	64	0.25	0.5	+	−	−	+	−	−	H
LF27	32	0.12	128	128	>128	64	16	1	+	−	−	+	−	−	H
LF61	64	32	128	>128	>128	128	0.5	1	+	−	−	+	+	+	H
LF68	64	32	128	>128	>128	64	0.25	1	+	−	−	+	+	−	H
LF83	64	32	>128	>128	>128	64	0.25	1	+	−	−	+	+	−	H
LF85	32	16	>128	>128	>128	128	0.25	1	+	−	−	+	+	−	H
LF91	64	32	>128	>128	>128	128	0.25	1	+	−	−	+	+	−	H
LF50	64	32	>128	>128	>128	64	0.5	0.5	−	−	−	+	+	+	H2
LF62	64	32	128	>128	>128	64	0.5	1	−	−	−	+	+	−	H2
LF40	64	32	128	>128	>128	64	0.25	0.5	−	−	−	+	+	−	H3
LF114	32	0.5	32	128	32	32	0.25	1	−	−	−	+	−	−	I
LF24	32	0.5	64	128	>128	64	0.25	0.5	−	−	−	+	−	−	I
LF26	32	0.25	32	128	>128	64	0.25	1	−	−	−	+	−	−	I
LF66	32	32	16	16	64	64	0.25	1	−	−	−	+	−	−	I
LF116	32	0.5	64	128	64	64	0.25	1	−	−	−	+	+	−	J
LF37	32	0.032	0.12	32	0.12	64	0.06	0.02	−	−	−	+	−	−	J
LF52	32	0.032	0.12	8	0.012	64	0.002	0.002	−	−	−	+	−	−	J
LF19	64	0.5	16	32	128	>128	0.25	1	+	−	−	+	−	−	J
LF21	32	32	64	64	128	64	0.25	0.5	−	−	−	−	−	−	K
LF14	32	32	32	64	>128	64	0.25	0.5	+	−	−	−	+	−	K
LF42	64	>128	0>128	>128	>128	64	>128	1	+	−	−	+	−	+	L
LF103	32	0.25	32	128	32	32	0.25	1	−	−	−	+	+	−	M
LF110	16	0.5	32	64	32	8	0.25	1	−	−	−	+	−	−	P
LF60	16	0.25	32	64	32	64	0.5	1	−	−	−	+	−	−	P
LF2	32	0.25	64	128	>128	64	0.25	0.5	−	−	−	+	−	−	P1
LF51	32	0.032	0.12	128	0.25	64	0.12	0.25	−	−	−	+	−	−	P1
LF1	32	0.5	64	128	>128	64	0.25	1	+	−	−	+	−	−	P1
LF13	64	0.5	128	>128	>128	64	0.25	1	+	−	−	+	−	−	P1A
LF35	32	0.12	32	32	>128	16	0.25	0.5	−	−	−	+	−	−	P2
LF72	16	0.25	16	64	32	64	0.25	1	−	−	−	+	+	−	P2
LF97	32	0.25	32	64	64	16	0.25	2	−	−	−	+	+	−	P2
LF106	32	0.25	16	64	32	128	0.25	1	−	−	−	+	−	−	P3
LF112	32	0.5	32	128	64	128	0.25	1	−	−	−	+	−	−	P3
LF113	32	0.5	32	128	64	64	0.25	1	−	−	−	+	−	−	P3
LF115	32	0.5	32	>128	64	128	0.25	1	−	−	−	+	−	−	P3
LF118	32	0.5	32	128	32	128	0.25	1	−	−	−	+	−	−	P3
LF46	32	0.12	16	32	128	64	0.5	0.5	−	−	−	+	−	−	P3

Continued

Table 2. *Continued*

Isolate	MIC (mg/L)								Antiseptic resistance genes						PFGE type
	AMP	GEN	OXA	CTX	CEF	CIP	TET	VAN	*norA*	*qacG*	*qacH*	*blaZ*	*smr*	*qacA/B*	
LF48	32	0.12	0.12	16	0.5	64	0.12	0.25	−	−	−	+	−	−	P3
LF49	32	0.12	2	16	16	32	0.25	0.5	−	−	−	−	−	−	P3
LF56	32	0.25	32	32	128	64	0.25	1	−	−	−	+	−	+	P3
LF6	32	0.25	64	128	>128	64	0.25	0.5	−	−	−	+	−	−	P3
LF69	16	0.25	32	64	64	128	0.5	2	−	−	−	+	+	−	P3
LF7	32	0.25	>128	>128	>128	128	0.25	1	−	−	−	+	−	−	P3
LF77	16	0.5	8	64	32	64	0.25	1	−	−	−	+	+	−	P3
LF90	32	0.25	64	128	64	16	0.25	1	−	−	−	+	+	−	P3
LF96	64	0.25	16	64	32	128	0.25	1	−	−	−	+	−	−	P3
LF99	16	0.5	32	64	32	128	0.25	1	−	−	−	+	−	−	P3
LF120	32	0.5	32	128	128	128	0.25	1	+	−	−	+	−	−	P3
LF30	32	0.032	0.12	16	0.5	64	0.12	0.5	+	−	−	+	−	−	P3
LF108	32	0.5	32	128	64	128	0.25	1	−	−	−	+	+	−	P4
LF87	64	0.5	>128	>128	>128	128	0.25	2	−	−	−	+	+	−	P4

AMP, ampicillin; CTX, cefotaxime; VAN, vancomycin; GEN, gentamicin; CEF, cefuroxime; TET, tetracycline; OXA, oxacillin; CIP, ciprofloxacin.

Biocide resistance genes and PFGE types

Table 3 shows the percentage of each biocide resistance gene present in the most common types and demonstrates whether the presence of biocide resistance genes relates to a specific PFGE type. *norA*, *smr* and *qacA/B* genes were detected with higher frequency in PFGE type EMRSA-16 than in EMRSA-15. Only two (11.1%) PFGE type EMRSA-16 isolates harboured *qacH*.

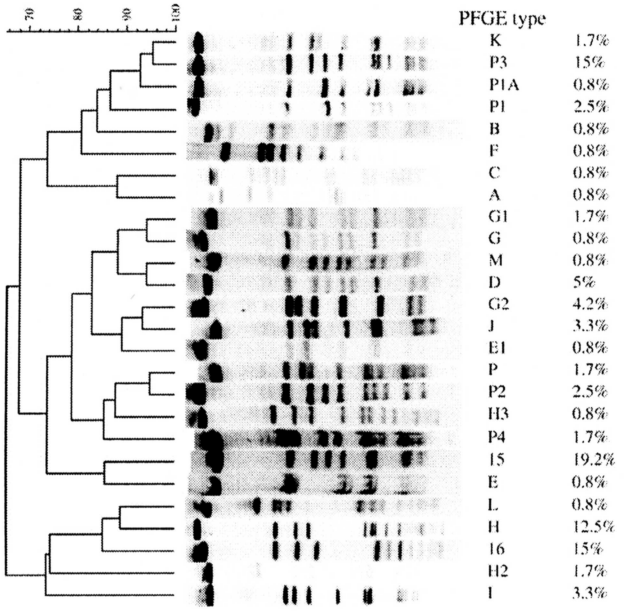

Figure 1. Percentage frequencies of the PFGE types and the phylogenetic analysis of MRSA isolates by PFGE gel patterns. Following restriction with *Sma*I, Dice similarities were subjected to cluster analysis as unweighted matched-pair groups.

Controls for surface disinfection and biocide residue tests

Comparison of counts with and without the use of a neutralizer showed that the neutralizer successfully quenched the effect of chlorhexidine at all biocide concentrations examined, with no significant difference between the counts with quenched chlorhexidine compared with a control with water alone ($P > 0.05$). Similarly, comparison of the counts with neutralizer compared with water alone showed that the neutralizer had no significant toxicity to the bacteria ($P > 0.05$).

Surface disinfection test

Figure 2 shows the mean ME of chlorhexidine when calculated using a drying control, specific to each strain and each time point. This was an attempt to take account of the effect of drying upon bacterial cell survival when calculating the ME. After 24 h, chlorhexidine has a lower impact on the clinical strains (mean ME = 0–0.61) than on the control strains (mean ME = 0.57–1.91) as shown by the lower range of mean MEs observed. Figure 3 shows the mean log cfu/mL of the controls with and without drying, and indicates that drying affects bacterial cell survival at 24 h drying time. After 24 h of drying, the reduction in log cfu/mL was more pronounced for the control strains than the clinical isolates, with mean reductions of 4 log cfu/mL observed in the control strains (EMRSA-16), with a maximum mean reduction of over 2 log cfu/mL in the clinical isolates (LF93).

Biocide residue test

Figure 4 shows the mean MEs of residues of chlorhexidine (2.5 mg/mL) dried for 2, 24 and 48 h against standard strains and clinical isolates. Generally the efficacy of chlorhexidine against the isolates decreased with longer biocide residue drying times. The residues exerted a similar minimal effect on both the standard strains and clinical isolates.

Table 3. Frequency of resistance genes within the most prevalent PFGE types

Resistance gene	PFGE type			
	15	16[a]	H[a]	P3
norA	34.8% (8/23)	66.7% (12/18)	66.7% (10/15)	11.1% (2/18)
smr	30.4% (7/23)	83.3% (15/18)	66.7% (10/15)	16.7% (3/18)
qacA/B	4.3% (1/23)	11.1% (2/18)	13.3% (2/15)	5.6% (1/18)
qacH	0% (0/23)	11.1% (2/18)	0% (0/15)	0% (0/18)

[a]PFGE types 16 and H are closely related at 85.15% percentage similarity. Similarity is based on the comparison of profiles using the Dice coefficient, preparation of a phylogenetic tree and cluster analysis using the hierarchic unweighted pair arithmetic average algorithm with an optimization of 1.0% and a tolerance of 1.0%.

Post-exposure MICs

The MICs for isolates were examined following exposure to chlorhexidine residues. Increases were observed in the MICs of cefotaxime, vancomycin, gentamicin, cefuroxime and oxacillin against the EMRSA-16 standard strain following 48 h of residue drying time (Table 4). There were also increases in the MICs of all tested antibiotics for the NCTC 6571 (*S. aureus* susceptible) strain following exposure to chlorhexidine residues that had been drying for 48 h (compared with the MICs for the strain before exposure).

Discussion

smr, *norA* and *blaZ* genes were widely distributed among *S. aureus* isolated from hospitals in Edinburgh. The presence of these genes provides increased tolerance to disinfecting agents[15] and the opportunity for the bacteria to survive antibiotic pressure.[23,24] In this study, *smr* was the predominant resistance gene, not *qacA/B*,[18,25] and also the concomitant presence of *qac* and *smr* was higher than previously reported,[25] although *qacA/B* confers resistance to a broader range of biocides than *smr*.[15,26,27] In general, resistance genes were independently detected among different PFGE types suggesting that these determinants are not specific to certain PFGE types and that reduced susceptibility to biocides may occur in any clinical *S. aureus* isolate. However, isolates harbouring both *qacA/B* and *smr* genes were either PFGE EMRSA-16 or closely related. Overall, in this study, strains representing PFGE type EMRSA-16 harboured more biocide resistance genes than EMRSA-15 or other PFGE types.

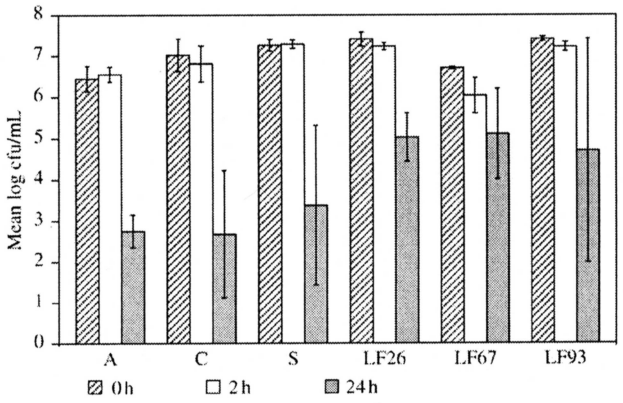

Figure 3. Mean log cfu/mL of non-drying control and the 2 and 24 h drying controls for each sample. A, *A. baumannii* ATCC 19606; C, EMRSA-16; S, *S. aureus* NCTC 6571; LF26, 67 and 93, other clinical MRSA isolates.

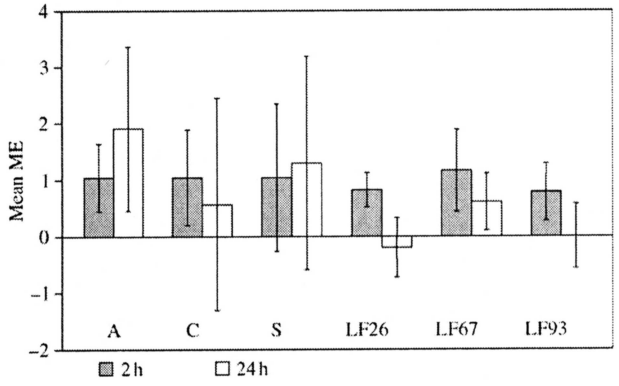

Figure 2. Mean ME of chlorhexidine after 2 and 24 h bacterial drying times, calculated compared with 2 and 24 h drying controls, respectively. A, *A. baumannii* ATCC 19606; C, EMRSA-16; S, *S. aureus* NCTC 6571; LF26, 67 and 93, other clinical MRSA.

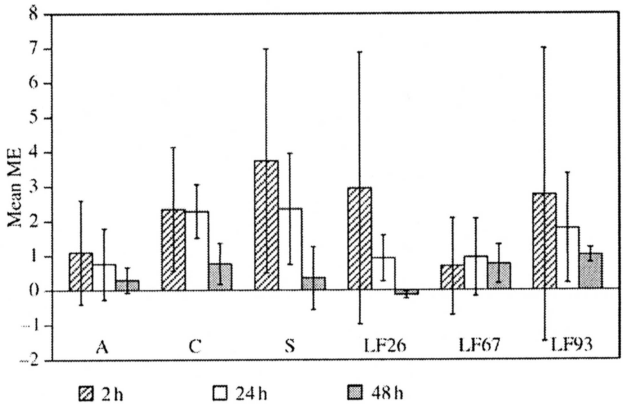

Figure 4. Mean ME of chlorhexidine after 2, 24 and 48 h biocide drying times. A, *A. baumannii* ATCC 19606; C, EMRSA-16; S, *S. aureus* NCTC 6571; LF26, 67 and 93, other clinical MRSA isolates.

Effect of chlorhexidine on MRSA

Table 4. Post-chlorhexidine-exposure MICs (mg/L) for EMRSA-16 and the susceptible control *S. aureus* NCTC 6571 after various biocide drying times

	Hours of drying	AMP	CTX	VAN	GEN	CIP	CEF	TET	OXA
EMRSA 16	control (no exposure)	>128	8	1	0.5	1	8	2	4
	2	>128	8	1	0.5	2	8	2	8
	24	>128	8	1	0.5	2	4	2	4
	48	>128	16	128	2	2	64	2	128
NCTC 6571 (susceptible *S. aureus*)	control (no exposure)	0.06	1	1	0.25	0.25	4	0.5	0.12
	2	0.06	1	1	0.5	0.25	1	0.25	0.12
	24	0.002	1	0.002	0.25	0.002	0.002	0.002	0.002
	48	128	32	>128	2	2	64	1	128

AMP, ampicillin; CTX, cefotaxime; VAN, vancomycin; GEN, gentamicin; CEF, cefuroxime; TET, tetracycline; OXA, oxacillin; CIP, ciprofloxacin.

Of interest was LF93 (PFGE type EMRSA-16); it contained *norA*, *blaZ* and *smr* genes, which when expressed may confer less susceptibility to quinolones and β-lactams as well as QACs, but not necessarily to chlorhexidine as one would expect with *qacA/B*.

Of note is that the MICs of oxacillin, cefotaxime, cefuroxime and ciprofloxacin for the four isolates with the *qacH* gene were high (≥128 mg/L) only when *norA* was also present. Similar to previous reports,[14,20] all the isolates that harboured the *qacA/B* gene also contained the β-lactamase transposon, *blaZ*. The close association between *qacA/B* and *blaZ* is common and represents a reservoir of resistance genes that can be transmitted to other strains.[20,26,28] However, not all isolates with *blaZ* contained *qacA/B*. The clinical implications of this are that antibiotic resistance does not necessarily encode biocide resistance; in contrast, reduced susceptibility to biocide is more likely to encode for antibiotic resistance.

There was a low efficacy of chlorhexidine against either standard strains or clinical isolates after 2 h of bacterial drying time. The efficacy was much increased against the standard strains after 24 h of bacterial drying time and increased to a lesser extent against the clinical isolates. It should be noted that drying has an effect on bacterial cell counts even without the presence of chlorhexidine, and attempts were made to take this into account.

The effectiveness of chlorhexidine residues upon bacterial suspensions decreased with longer drying times. However, even after 48 h, the residues still exerted an effect on most isolates. Because this effect was minimal, it therefore may act as a selective pressure and allow the less susceptible strains to persist in the clinical environment by incomplete eradication. Such a situation may occur in the hospital environment where exposure to low or residual concentrations of biocides may persist on surfaces leading to reduced efficacy. Although biocides when used at concentrations instructed by the manufacturers are bactericidal, concentrations that might remain on surfaces after cleaning might provide a selective pressure on microorganisms. In theory, sublethal concentrations of biocide for any given cellular target may occur at some point along this concentration gradient, providing a selective pressure for mutations in a range of cellular targets.[29]

The varying effect of chlorhexidine upon clinical isolates, as observed in both the surface disinfectant and biocide residue tests, is of importance as it may mean that certain isolates will have an ability to survive chlorhexidine treatment and that the use of biocides could act as a selective pressure to allow these isolates to predominate.

The increases in the MICs of all tested antibiotics for the susceptible control *S. aureus* strain following exposure to surface dried chlorhexidine residues is of interest as it suggests that the use of chlorhexidine in the hospital environment may be linked to increased resistance to antibiotics in previously susceptible strains. The exposure to subinhibitory doses of biocides selects for up-regulation of efflux pumps capable of transporting these compounds as well as some antibiotics out of the cell and contributes to reduced biocide susceptibility.[30] It may be that the long period of surface drying of chlorhexidine leads to reduced efficacy of the biocide, thus allowing the persistence of isolates when the biocide is left as a residue.

Biocides are critical components of intervention strategies used in clinical medicine for preventing the dissemination of nosocomial infections. Reduced susceptibility to biocides and the threat this represents is a serious concern. It is important to determine the susceptibility of clinical MRSA to various biocides to assess the control and preventive measures currently implemented in hospitals.

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Transparency declarations

None to declare.

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